THE IMPACT OF TESTOSTERONE ON MURINE MODELS OF INFECTION AND VACCINATION

By

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ABSTRACT

In men, low testosterone is associated with an increased risk of all-cause and cardiovascular related mortality, and although its immunomodulatory properties have been well characterized, the impact of testosterone on both the severity of viral infection and the efficacy of vaccination remain poorly understood. The severity of influenza increases with age in men, and as circulating testosterone concentrations also decline with age in men, I hypothesized that reduced testosterone contributes to age-associated increases in influenza severity. A murine model was used, and consistent with in humans, young male mice had greater testosterone concentrations than did aged males. Following IAV infection, aged male mice experienced greater disease severity, mortality, and pulmonary inflammation than young males, while control of viral replication was delayed. Removal of testosterone in young males increased disease severity, and pulmonary inflammation independent of changes in viral replication. Because testosterone in young male mice reduced pulmonary inflammation, I further hypothesized that testosterone was altering the immune response to IAV infection. Testosterone reduced IAV severity not by changing pulmonary cytokine activity, but instead by accelerating pulmonary leukocyte contraction. To identify which immune cell types were persisting in testosterone-depleted males, we further characterized the composition of pulmonary cellular infiltrates. Testosterone depletion accelerated the contraction of IAV-specific $CD8^+$ T cells, while inhibiting the influx of eosinophils into the lungs following clearance of virus from the lungs. The effects of testosterone on IAV-specific $CD8^+$ T cells were mediated androgen receptor signaling and dependent on the environment in which they reside. In contrast with IAV infection where the immune suppressive effects of testosterone are protective, in the context of vaccination any reduction in immune response may be detrimental. Adult females tend to develop greater adaptive immune responses than males following vaccination in both preclinical

animal studies and human clinical trials. Following vaccination with irradiated transgenic *P. berghei* sporozoites expressing the *P. falciparum* CSP protein, adult female mice mounted greater adaptive immune responses and were better protected against challenge than adult males. No sex differences in adaptive immune responses or protection were observed in mice vaccinated prior to puberty, suggesting a role for sex steroid hormones. Depletion of testosterone in males increased, whereas rescue of testosterone decreased, adaptive immune responses and protection in males following parasite challenge. Taken together, these data suggest that testosterone confers protection during IAV infection by modulating the immune response, while testosterone concentrations in males reduce adaptive immunity and contributes to reduced malaria vaccine efficacy.

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ARDS	Acute respiratory distress syndrome
AR	Androgen receptor
ARE	Androgen response element
cDC	Conventional dendritic dell
CSP	Circumsporozoite
DC	Dendritic cell
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
GABA-A	γ-aminobutyric acid type A
GnRH	Gonadotropin-releasing hormone
HA	Hemagglutinin
HIV	Human immunodeficiency virus
HPG	Hypothalamic-pituitary-gonadal
IAV	Influenza A virus
IBV	Influenza B virus
ICV	Influenza C virus
IL	Interleukin
ILC2	Type II innate lymphoid cell
IP	Intraperitoneal
IV	Intravenous
KO	Knock-out
LH	Luteinizing hormone
NHP	Nonhuman primate
NA	Neuraminidase
NK	Natural Killer
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PPAR	Peroxisome proliferator-activated receptor
RNP	Ribonucleoprotein
SA	Sialic acid
SHBG	Sex-hormone-binding globulin
$TCID_{50}$	Tissue culture infectious dose 50
Tfm	Testicular feminized mice
TNFα	Tumor necrosis factor α
TRAIL	TNF-related apoptosis inducing ligand
Tregs	Regulatory T cells

Chapter 1

INTRODUCTION

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Introduction to testosterone

In addition to its better-known role as the primary male sex hormone, testosterone has been shown to have profound effects on a wide range of biological processes and influence states of health and disease in both sexes [1-3]. Testosterone along with dihydrotestosterone (DHT), androstenedione, and dehydroepiandrosterone (DHEA) comprise the androgen family of steroid hormones [4]. Of these four, testosterone is considered the dominant androgen and is found at the highest concentration in the circulation [5]. Though of higher potency, DHT is found at significantly lower concentrations and is irreversible converted from testosterone by the enzyme 5 α-reductase, with the liver being the primary site of testosterone's conversion to DHT [4-7]. Both DHEA and androstenedione are produced by the adrenal gland and exert 1/10 and 1/20 the androgenic effects of testosterone respectively [6, 8]. Though both can be converted to testosterone, the effects of this process on circulating testosterone concentrations is minimal [6, 8]. Of the androgens, only DHT is unable to be converted to estrogen, thus simplifying the interpretation of DHTs effects on biological processes [4-6].

In males, testosterone is primarily produced by the Leydig cells of the testes under the control of the hypothalamic-pituitary-gonadal axis (HPG) [8-10]. With the HPG axis, the hypothalamus first releases gonadotropin-releasing hormone (GnRH) in a pulsatile fashion which stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) into the circulation from the anterior pituitary gland [8, 9]. LH stimulates the Leydig cells of the testes to produce testosterone from cholesterol, while FSH acts on the Sertoli cells of the testes to stimulate spermatogenesis in the presence of testosterone [8, 9]. The production of testosterone is regulated by negative feedback, with high levels of testosterone resulting in the suppression of LH and GnRH secretion [8, 9]. Testosterone production by the Leydig cells results

in a relatively high concentration of testosterone in the testes which is then released into the periphery, yielding lower tissue concentrations of testosterone [8].

In the male periphery, only 2% of testosterone is typically found in an unbound state, 30% is bound at high affinity to sex-hormone-binding globulin (SHBG), and the remainder is bound at relatively low affinity by non-specific proteins including albumin [6, 8, 11]. Free testosterone refers to the fraction of testosterone unbound to plasma proteins, while bioavailable testosterone refers to fraction of circulating testosterone that is either unbound or bound at low affinity by albumin and is thus able to readily dissociate in the tissues [8, 12]. It is this free state of testosterone that is believed to the biologically active form of testosterone [13, 14]. Total testosterone refers to both the unbound and bound fractions of testosterone [8, 12]. In humans, both albumin and SHBG play important roles in regulating the bioactivity, metabolism, transport, and tissue specific localization of testosterone, with SHBG being shown to inhibit androgen bioactivity in vivo and in vitro [12-14]. Whether SHBG is similarly involved in the regulation of androgens in adult rodents is unclear. Though hepatic expression of SHBG has been detected in fetal rats, only trace amounts have been detected in the serum of adult rats and mice [15, 16].

Most biological activities of testosterone and the other androgens are entirely mediated through the actions of the androgen receptor (AR) through a variety of mechanisms [2, 5, 17-19]. The AR is a member of the nuclear receptor superfamily and is encoded on the X chromosome [2, 17, 20]. In the canonical or genomic pathway of androgen receptor activation, the AR functions as a ligand-dependent nuclear transcription factor [2, 17, 18]. In the absence of ligand (e.g., testosterone or DHT), the AR typically resides in the cellular cytoplasm in association with heat shock proteins (HSP) including HSP90, HSP70, and p23 [2, 17, 18, 21]. Androgens can freely diffuse through the plasma membrane and binding to the AR induces a conformational

change, resulting in heat shock protein dissociation, nuclear translocation, and the dimerization of the AR [18]. Once in the nucleus, co-activators are recruited, and the dimerized AR-ligand complex recognizes and binds to specific DNA sequences known as androgen response elements. Following ARE binding, the entire complex then functions to regulate the transcription of androgen-responsive genes [17, 18]. In addition to this genomic pathway, the AR is also able to activate non-genomic signaling pathways, which are characterized by rapid androgenic responses resulting in cellular changes without the need to first alter transcription and translation of androgen responsive genes [18]. The non-genomic AR signaling pathways are generally considered to originate at either the plasma membrane or the cytoplasm and can result in the release of intracellular calcium or the activation of protein kinases including mitogen-activated protein kinase, protein kinases A, B, and C [18, 19, 22]. Cellular expression of AR generally considered to be needed for direct androgenic effects on cells, and genetic models have demonstrated widespread mammalian AR expression throughout a variety of cell and tissue types including those of immune system [17, 23, 24].

Testosterone deficiency and androgen replacement therapy

In males, after an initial surge during fetal development, testosterone concentrations remain low and then rapidly increase with the onset of puberty [25]. In normal males, testosterone production peaks at roughly 20-30 years of age and thereafter gradually declines with progressing age, with clinically low testosterone levels observed in 19% of men in their 60's increasing to 49% of men in their eighties [26-28]. By the year 2025, as many as 6.5 million American males 30-79 years of age are projected to meet the clinical definition of symptomatic testosterone deficiency [29]. The reasons for age-related declines in androgenic function are believed to involve decreased Leydig cell responsive to LH stimulation leading to a reduction in

total testosterone production [30, 31], as well as age-associated increases in SHBG [32-34]. In addition to age related declines in testosterone, genetic conditions (e.g., Klinefelter's syndrome and congenital gonadotropin deficiency), medically induced hypogonadism, and tissue damage from infection (e.g., mumps associated orchitis) or trauma can result in low testosterone concentrations in otherwise reproductively aged males [9].

In addition to its it's well know role as the primary males sex hormone, testosterone can have profound effects on systemic health and disease and has been demonstrated to modulate the functions and activities of a variety of tissue and cell types [35]. For example, in addition to regulating sperm production and secondary male sex-characteristics, testosterone has also been shown to influence metabolic function, cardiovascular function, promote bone mineral density, and alter the functions of the immune system [25, 36-39]. Consistent with these observations, men with low testosterone, whether congenital, acquired, or age-related, are at an increased risk of all-cause and cardiovascular related mortality [35, 40-43]. Additionally, low testosterone in adult males has been associated with metabolic dysfunction, osteoporosis, muscle weakness, fatigue, cognitive impairment, and sexual dysfunction; while in hypogonadal men, testosterone replacement therapy has been shown to improve cardiovascular disease outcomes, increase quality of life perceptions, and improve age-associated anemia [41, 44-48].

Although safety concerns exist (e.g., cardiovascular disease risk) [49, 50], the perceived consequences of low testosterone combined with the potential benefits of testosterone replacement therapy have resulted in a dramatic increase in its therapeutic use over the last two decades [49-52]. As of 2013, an estimated 2.3 million men were undergoing testosterone replacement therapy in the United States alone in 2013, representing 1.67% of the insured male population aged 30 and older, with the highest frequency of usage occurring in men 40-64 years of age [49, 50]. Included in these numbers is a 4-fold increase in testosterone replacement

therapy use in reproductively aged males (i.e. 18 to 45 years of age), a demographic often overlooked in studies of the implications of low testosterone [53]. However, despite the increasing popularity of testosterone replacement therapy, little remains known of the influence of testosterone deficiency and testosterone replacement therapy on the outcomes of infection and vaccination.

The immunomodulatory effects of testosterone

Testosterone and DHT have been shown to be broadly immunomodulatory and capable of significantly altering the cellular functions of both innate and adaptive immune cells, either through direct AR signaling in target cells, or indirectly through interactions with other cells types or the environment in which they reside [5, 23, 54-57]. Furthermore, developmental stage specific AR expression and androgenic effects have been described for immune cells, complicating the interpretation of androgenic effects on the immune system [54, 58, 59]. When acting on the immune response, testosterone in generally considered to be immunosuppressive [23], and the known effects of testosterone on the major cell populations of the immune system are summarized below.

Both human and murine neutrophils have been shown to express functional ARs [24, 60], with the numbers of mature neutrophils and the proliferative potential of bone-marrow derived neutrophil precursors being reduced in AR KO mice [60]. In both mice and in humans, testosterone treatment has been shown to promote tissue recruitment and accumulation of neutrophils while altering their functional activities [61, 62]. For example, testosterone is associated with reduced neutrophil expression of proinflammatory mediators including decreased expression of CD11b, reduced production of reactive oxygen species, and reduced

leukotriene biosynthesis, while testosterone promotes expression of anti-inflammatory mediators including IL-10 and TGFβ [61, 62].

As with neutrophils, both human and murine macrophages populations have been shown to widely express functional ARs, with testosterone having been shown to broadly alter the biological activities of these cells [24, 63-66]. In both mice and humans, testosterone and AR signaling in macrophages has been shown to suppress the production of proinflammatory mediators (e.g., nitric oxide, IL-β, IL-6, and TNFα) while promoting the production of IL-10 [67-70]. In murine models of allergic lung inflammation, AR signaling in males promotes M2 macrophage polarization, while the expression of the eosinophil recruiting chemokines *Ccl11* and *Ccl24*, were reduced in AR-deficient murine alveolar macrophages [71]. Gonadectomy of male mice increased macrophage expression of TLR4 [72], while reducing the number of splenic macrophages in Balb/C mice [73]. Testosterone has also been shown to inhibit apoptosis of bone-marrow derived macrophages of *Leishmania donovani* infected female mice [74]. Moreover, both testosterone and DHT treatment have been shown to reduce Fcγ receptor expression by splenic macrophages in guinea pig models of *Leishmania* infection [75].

Dendritic cells (DCs) are a diverse pool of professional antigen presenting cells that canonically play roles in immune surveillance, cytokine production, and the activation and orchestration of T cell responses [76-78]. Two predominant DC subsets have been identified: conventional dendritic cells (cDCs) which typically specialize in immune surveillance and antigen presentation to T cells, and plasmacytoid dendritic cells (pDCs) which are characterized by a reduced capacity for antigen presentation while being the dominate source of both type 1 and type 3 interferons following viral infection [23, 76, 77, 79]. Despite, the lack of reported androgen receptor expression by both pDCs and cDCs, androgenic effects on DC populations have been observed in studies of both humans and in mice [54]. In castrated mice, depletion of

testosterone has been shown to promote cDC maturation and increase costimulatory molecule (e.g., MHCII, CD80, and CD83, CD86, and OX-40L) expression [80]. cDCs in the peripheral blood of hypogonadal men show increased activation following TLR9 signaling with expression of the activation and degranulation marker CD107b being inversely correlated with serum testosterone concentration [81]. Furthermore, clinical studies have demonstrated reduced pro-inflammatory cytokine production (e.g., IL-1β, IL-6, and TNFα) by cDCs following testosterone treatment [79]. Treatment of human pDCs with DHT significantly reduced IFNα production following TLR7 activation [82]. Given the lack of functional AR expression by dendritic cells, the mechanisms mediating these androgenic effects are currently remain unknown.

Like DCs, eosinophils have been shown to be androgen-responsive despite the absence of functional androgen receptor expression [24, 54, 83]. In murine models of *Brugia pahangi* infection, testosterone has been shown to suppress eosinophilic responses to infection [84], while testosterone treatment decreases peripheral eosinophil counts in both castrated male and female guinea pigs [85, 86]. Furthermore, testosterone has been shown to reduce eosinophilic adhesion to human mucosal microvascular endothelial cells in the nasal mucosa and reduce tissue specific eosinophilic viability [87]. In mice, testosterone-mediated differences in eosinophilic responses have been attributed to the actions of Type II innate lymphoid cells (ILC2s), with AR signaling inhibiting the differentiation of ILC2 progenitor cells into mature ILC2s [88, 89]. Treatment of mice with DHT reduces ILC2 numbers and reduces the production of IL-5 needed for eosinophilic responses to airway inflammation [89].

The effects of testosterone and AR signaling on other innate immune cell populations are less well characterized. Testosterone treatment of murine natural killer (NK) cells results in reduced cytotoxic activity [90], while medical castration of human males increases the percentage of NK cells isolated from PBMCs relative to healthy controls [91]. Furthermore,

testosterone has been shown to reduce IL-6 production by monocytes isolated from human PBMCs [92, 93].

Though the effects of female sex hormones on the biological activities of B cells are well established, the effects testosterone on these cell populations are less well understood [94]. Male patients with Klinefelter's syndrome and idiopathic hypogonadotropic hypogonadism have elevated peripheral B cell counts relative to healthy controls, which are subsequently lowered following the initiation of androgen replacement therapy [95, 96]. In mice, bone marrow, splenic, and peripheral blood B cell populations expand following castration and are elevated in androgen insensitive Tfm mice [97-103]. In humans, higher testosterone concentrations are associated with reduced neutralizing antibody responses following vaccination with the trivalent inactivated seasonal influenza vaccine [104], while testosterone treatment of both total human PMBCs and purified B cells reduced total IgG and IgM antibody production in both systemic lupus erythematosus patients as well as in healthy controls [92, 93]. In neonatal gonadectomized rats infected with Seoul virus, treatment with DHT reduces anti-Seoul virus IgG and Th1 associated IgG2a antibody responses [105]. Despite the suppressive effects of testosterone on humoral immune responses, functional AR expression has not been consistently detected by mature peripheral B cell populations [5, 23, 59]. Instead, evidence suggests testosterone primarily regulates humoral immunity during lymphopoiesis prior to the acquiring of antigen specificity, with both immature B cell populations and bone marrow stromal cells having been shown to express functional ARs [5, 97, 103, 106]. Consistent with this idea, inhibition of AR signaling in osteoblast-lineage cells increase B cell lymphopoiesis in the bone marrow of male mice, while androgens have been shown to promote the apoptosis of immature B cells [94, 107]. However, one study has demonstrated that B cells isolated from the PBMCs of healthy donors not only expressed low levels of AR, but were also capable of metabolizing

androstenedione to testosterone via 17β-HSD expression and further capable of metabolizing testosterone to DHT via 5α-reductase in vitro [108]. Whether intracrine testosterone synthesis is biologically relevant process for lymphocytes, or an artifact of in vitro manipulation is unclear [109].

Testosterone has also been shown to alter the function, differentiation, and fate of both $CD4^+$ and $CD8^+$ T cell populations. In hypogonadal men, the proportion of $CD4^+$ T cells isolated from PBMCs is increased relative to healthy controls, while these numbers normalize following the initiation of testosterone replacement therapy [110]. In vitro, testosterone has been shown to arrest cell cycle progression and promote apoptosis in established T cell lines, while promoting Fas-dependent apoptosis of Coxsackie virus specific Th2 cells by decreasing Bcl2 expression [111, 112]. In mice, androgen ablation has been shown to increase the numbers of both $CD4^+$ and $CD8^+$ T cells, while increasing their proliferative potential in response to stimulation [113]. In animal models of infection and inflammation, androgens have been shown suppress T cell recruitment and the production of IFNγ, IL-2, and IL-12 by T cells, while promoting the expression of Th2 and Th17 associated cytokines including IL-4, Il-5, and IL-17 [114-121]. Moreover, in both humans and in animal models, testosterone has been shown to promote the expansion of regulatory T cell (Tregs) populations, while enhancing IL-10 production and Foxp3 gene expression within these cells [114, 116, 118, 122-125]. In contrast with the generally anti-inflammatory effects of testosterone reported above, testosterone treatment of male mice infected with Coxsackievirus group B viruses enhances production of Th1 associated cytokines including IL-2 and IFNγ by splenic $CD4^+$ T cells, while testosterone treatment of female mice suppresses Th2 associated production of IL-4 and IL-5 by splenic $CD4^+$ T cells [126]. Whether, proinflammatory effects of testosterone in this model represents a difference in pathogen specific responses is unclear.

Despite the first paper showing a link between male castration and thymic enlargement being published in 1904 [127], the exact mechanisms by which testosterone alters the biological activities of T cells remains unclear. While some groups have failed to detect functional ARs in mature T cells isolated from mice, humans, and established T cell lines [97, 125, 128, 129], others have reported functional cytosolic AR expression and demonstrated functional genomic AR signaling in these cells [108, 123]. Furthermore, other groups have suggested that murine T cells only express membrane bound ARs with androgenic effects being mediated through non-genomic calcium release [55, 116, 130-133]. The most convincing body of evidence supports a role for testosterone in regulating T cell selection and development in the thymus. As was first reported over 100 years ago, castration of mice results in enlargement of the thymus and increases thymic output, while testosterone replacement in castrated mice results in thymic regression and decreases the number of $CD4^+CD8^+$ double positive thymocytes [23, 127, 134, 135]. Furthermore, androgens have been shown to both directly and indirectly lead to thymocyte apoptosis with thymic T cells being shown to express functional AR and exhibit classical AR signaling in response to androgen stimulation [97, 131, 136-138]. Moreover, both the cortical and medullary regions of the thymus express AR and respond to androgen treatment [97, 139, 140], with AR signaling in thymic epithelial cells being shown to increase positive selection of thymocytes [141, 142].

The effects of testosterone on Infection and vaccination

Despite the well-established body of literature showing profound immunomodulatory activities of testosterone, relatively little is known about the influence of testosterone on the outcomes of infection and vaccination. Moreover, though sex differences in infectious disease outcomes in have been well documented, and age-related changes in disease risk have been

postulated to be mediated by testosterone changes, surprisingly few human epidemiological studies have directly evaluated the role of testosterone as a variable mediating these differences [56, 143-148]. In those studies where androgen concentrations were considered, high testosterone concentrations have been correlated with the increased prevalence of human papilloma virus in men who have sex with men [149], while free testosterone concentrations in women, but not, men have been associated with throat colonization of *Staphylococcus aureus* [150]. In community-acquired pneumonia patients, testosterone concentration has been shown to be inversely correlated with the severity of pneumonia and organ failure risk [151], while hypogonadism due to Klinefelter's syndrome is associated with increased pneumonia associated mortality [43]. Furthermore, low serum testosterone is associated with an increased risk of morbidity and frailty in males infected with human immunodeficiency virus (HIV) [152], as well as an increased risk of infection related hospitalization in male hemodialysis patients [153].

Most direct evidence for a role in testosterone in altering the outcomes of infectious diseases comes from the use of animal models. In rodent models of amebic infection, testosterone concentrations correlate with the incidence of amebic liver abscesses, with testosterone increasing the severity of *Entamoeba histolytica* infection at least in part through inhibition of IFNγ production by natural killer T cells [114, 143, 154]. In mice infected with *Babesia microti*, testosterone is associated with increased parasitemia and anemia [155], while the severity of *Toxoplasma gondii* infection is reduced in female mice following testosterone treatment [156]. Following *Leishmania major* infection, male mice typically have greater hepatic parasite loads than females, with these differences being reversed through castration of males or testosterone treatment of females [157, 158]. In mice infected with *Brugia pahangi*, testosterone treatment of either castrated males or female mice, reduced resistance to

infection and suppressed intraperitoneal lymphocyte, macrophage, and eosinophilic responses [159].

Moreover, these effects of testosterone are not limited to parasitic infections. In murine models of *Mycobacterium marinum* infection, skin lesion severity and the frequency of systemic bacterial dissemination is greater in male mice relative to females with these differences being reversed through testosterone depletion in males [160, 161]. Baseline testosterone concentrations have been correlated with viremia following experimental infection with Venezuelan equine encephalitis virus in macaques [162], while early work on the effects of testosterone on influenza A virus (IAV) infection in female swiss mice found that testosterone treatment increased viral loads and the rate of viral replication in the lungs [163]. In contrast, more recent work in lethal models of IAV infection, have shown that surgical testosterone depletion in male mice significantly increases morbidity and death, but does not alter either the magnitude or the kinetics of viral replication [164].

As with infection, though sex differences in the outcome vaccination have been well described [57, 165, 166], only a handful of studies have evaluated the impacts of testosterone on the immune response to vaccination. Notably, in humans, elevated serum concentrations are associated with reduced neutralizing antibody responses to the trivalent inactivated influenza vaccine [104]. In HIV infected men who received the seasonal influenza vaccine, plasma testosterone concentrations have been shown to be inversely correlated with influenza-specific antibody avidity [167]. While, testosterone treatment of female mice suppresses protection following vaccination with the surface membranes of *P. chabaudi* infected red blood cells [168]. Finally, though testosterone replacement therapy in aged male macaques has been shown to reduce the decline in immune function, only modest improvements in antibody production following vaccinia virus vaccination were observed [169].

Taken together, these data suggest that, testosterone is highly immunomodulatory and can have profound influences on the outcomes of infection, inflammation, and vaccination. Whether these effects are beneficial or detrimental will likely be dependent on the nature of the immunological challenge. I hypothesize that testosterone will be protective in instances of infection where disease is largely mediated by the immune response to the pathogen (e.g., IAV), while detrimental to the development of protective immune responses following vaccination.

Influenza A viruses

Background

Influenza viruses occupy four genera of the *Orthomyxoviridae* family and are classified as influenza A (IAV), B (IBV), C (ICV), or D (IDV) viruses based on antigenic variation of the nucleoprotein (NP) [170, 171]. All influenza viruses are enveloped and possess a segmented single stranded negative sense RNA genome, with IAV and IBV comprised of 8 segment genomes, while ICV and IDV are comprised of 7 segment genomes [172]. For IAV and IBV, the viral surface contains two primary glycoproteins, the hemagglutinin (HA) protein that facilitates viral entry via the sialic acid receptor (SA), and the neuraminidase protein (NA) that is involved in viral release [170], while for ICV and IDV, both functions are replaced by the single hemagglutinin-esterase-fusion protein [173]. The viral envelope also contains the ion channel forming matrix 2 protein (M2), while the matrix 1 protein (M1) forms a coat just under the viral envelope [172]. Inside this structure resides the viral ribonucleoprotein complexes (RNP) which are comprised of each of the genomic negative sense RNAs encapsulated in NP protein along with each of the three polymerase proteins (PA, PB1, and PB2) [172]. Influenza viruses also contain non-structural protein 1 (NS1), which functions as a viral virulence factor [174], and the nuclear export protein (NEP) which is primarily involved nuclear export of the viral RNP [175].

Due to their increased diversity, IAV isolates are further classified into subtypes based on the antigenic properties of the HA and NA surface proteins, with 16 HA (H1-H16) and 9 NA (N1-N9) subsets being isolated to date from waterfowl [172]. An additional two HA (H17 and H18) and two NA (N10 and N11) subsets have isolated from bats, but these isolates do not appear to recognize sialic acid (SA) or be capable of reassortment with other IAV isolates [170, 176]. Host specificity and tissue tropism is in part mediated by HA sialic acid binding preferences, with human isolates preferentially binding to α2,6 linked sialic acid residues, while

HA proteins of avian isolates preferentially bind to α2,3 linked sialic acid residues [170]. Because of the immense selective pressure placed by the immune system on the HA and NA proteins, antigenic drift (i.e., the gradual accumulation of mutations), continually yields new antigenic variants, which can be attributed to the error prone nature of the RNA-dependent RNA polymerase and the lack of proof-reading abilities by this structure [177]. Further IAV diversity results from less frequent antigenic shifts which are the result of genome reassortment following coinfection with dissimilar strains. This is more likely to occur in some species such as swine which express both avian and human receptor and can serve as mixing vessels, as was the cases with the 2009 pandemic strain which resulted from a triple reassortment [178].

Migratory waterfowl (order Anseriformes) and shorebirds (order Charadriformes) are the natural reservoir of influenza A viruses, with the virus being capable of infecting, and being transmitted, within numerous other species (e.g., domestic poultry, swine, seals, bats, dogs) including humans [170, 179, 180]. Though a cause of gastrointestinal infection within avian populations, influenza virus infection in mammals leads to mild to severe disease of the respiratory tract [170]. Cross-over of zoonotic IAV strains and subsequent adaptation to humans can result in emergence of worldwide influenza pandemics such as those seen with the 1918 Spanish flu (H1N1), the 1957 Asian flu (H2N2), the 1968 Hong Kong flu (H3N1), and the 2009 H1N1 pandemic strain [170, 178]. The most well-known of these pandemics was the 1918 Spanish flu, which was estimated to have infected roughly one third of the world population killing an estimated 50 million people [178]. Though somewhat less dramatic than influenza pandemics, seasonal influenza transmission causes a significant global burden of disease. In the United States, annual seasonal influenza virus transmission results in an estimated 115,000 to 630,000 hospitalizations and 5,000 to 17,000 deaths [181], while globally these numbers are estimated at 3 to 5 million cases of severe illness and roughly 500,000 deaths per year [170]. In

the Netherlands, influenza has been identified as the second leading cause of infection related disability-adjusted life year [182]. Influenza also has significant economic implications, with the direct and indirect economic cost associated with seasonal influenza estimated to be 11.2 billion dollars per year in the United States alone [183]. Currently, both the H1N1 and H3N2 subtypes, as well as two strains of IBV, circulate within the human population on a seasonal basis corresponding with the cold season in temperate climates, while seasonal forcing appears to be less defined in the tropics [170].

Innate immune response to primary influenza A virus infection

Influenza A viruses primarily infect and productively replicate in epithelial cells of the upper and lower respiratory tract [184-186], with these cells forming the first line of defense against influenza virus infection. In addition to physical and mechanical barrier functions (e.g., mucus production and ciliary action), these cells cell limit initial viral infection through the activation type I and type III IFN responses [187-190]. Infection of epithelial cells additionally results in the production of proinflammatory cytokines and chemokines that trigger the induction of the local and systemic inflammatory response. For example, early production of cytokines including IL-1, IL-6, and TNFα facilitate innate immune cell infiltration out of the circulatory system, while chemokines such as CXCL1 and CCL3 recruit neutrophils and macrophages to the site of infection [190].

Tissue resident alveolar macrophages are typically found within the luminal spaces of the respiratory tract (e.g., alveoli, bronchi, and bronchioles) where they perform immune surveillance functions and help maintain lung homeostasis [191]. During IAV infection, these cells are typically the first immune cells to encounter virus and contribute to viral clearance through the phagocytosis of virally infected apoptotic cells, the production of inflammatory

mediators including TNFα, IL-1β, CXCL10 and INOS, and the direct inhibition of viral infection of alveolar epithelial cells [192-196]. Additionally, alveolar macrophages have been shown to be critical mediators of antibody-induced inflammation and antibody-dependent cellular phagocytosis following IAV Infection [197]. In the absence of alveolar macrophages, IAV infection results in the accumulation of cellular debris and the loss of respiratory function, suggesting that these cells may additionally play a role in the in resolution of disease [198]. This is further supported by observations that alveolar macrophages can be induced to express markers of a M2 immunoregulatory phenotype, including expression of IL-10 and hepatocyte growth factor [191, 199, 200]. Though other macrophage populations are recruited to the sites of IAV infection, including interstitial macrophages which typically reside in the lung parenchyma, their contribution to the control of infection remains under studied [191, 201, 202].

Neutrophils are typically the earliest cell type to cross the vascular epithelium in appreciable numbers and localize in IAV infected tissues, where they contribute to the control of viral replication through the phagocytosis of apoptotic IAV infected cells, cytokine release, and the production of reactive oxygen species [54, 203]. Depletion of neutrophils in mice increases the severity of moderate to severe IAV infection, and leads to increased mortality, increased pulmonary viral load, viral dissemination to extrapulmonary tissues, and decreased lung function [204-207]. In addition to phagocytic functions, neutrophils play a critical role in the recruitment of $CD8^+$ T cells to the sites of IAV infection at least in part through the deposition of the leukocyte chemoattractant CXCL12 [208]. Consistent with these observations, early depletion of neutrophils in murine models reduces the magnitude of $CD8^+$ T cell infiltration and results in impaired $CD8^+$ T cell activation [209]. As with other cell types, excessive or aberrant neutrophil responses can enhance disease following IAV infection. For example, in clinical cases

of IAV, the presence of high levels of neutrophil extracellular traps correlates with lung damage and is associated with poorer clinical outcomes, while the number of neutrophils in the lower respiratory tract has been correlated with severe outcomes following IAV infection [210, 211]

Dendritic cells play several important roles in immunity to IAV infection and can be broadly divided into two categories: plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) (i.e., myeloid derived dendritic cells) [212, 213]. In addition to producing proinflammatory cytokines, DCs can be directly infected with IAV or otherwise traffic IAV peptide to the draining lymph nodes, where they present antigen to and prime $CD4^+$ and $CD8^+$ T cells more efficiently than macrophages populations [213, 214]. Following primed T cell migration to the respiratory tract, these cells further proliferate, differentiate, and develop effector phenotypes following further interactions with DC populations [213, 215]. Plasmacytoid dendritic cells require direct IAV infection to perform their APC and T cell support roles [216]. Protective IAV-specific $CD8^+$ T cell responses require interactions with dendritic cells in the lungs to maintain $CD8^+$ T cell populations (both pDCs and $CD8a^+$ populations [215].

The role of eosinophils in the immune response to IAV has been less well studied. Eosinophils are susceptible to IAV infection, and in murine models of asthma, activated eosinophils have been associated with increased numbers of $CD8^+$ T cells in the airways. Furthermore, IAV virus or peptide-exposure alone is enough to induce eosinophilic degranulation and induce $CD8^+$ T cell proliferation and activation [217]. Eosinophils can acquire and present IAV peptide [218]. Epithelial cells produce RANTES in response to IAV infection and has strong chemoattractant phenotype for eosinophils [219].

Adaptive immune response to influenza A viruses

The adaptive immune response to IAV consists of $CD4^+$ and $CD8^+$ T cell responses in addition to antibody production by B cells, with all three cell types playing critical roles in the efficient control of influenza virus infection [220]. Following IAV induced activation, $CD4^+$ T cells can differentiate into several Th subsets that carry out diverse effector. $CD4^+$ T cell differentiation in the presence of IL-6 and IL-21 leads to the development of T follicular helper cells (Tfh), which are involved in the development of B cell responses during influenza virus infection [221]. Differentiated Th1 cells produce IFN-γ, TNF-α and IL-2 and promote cytotoxic $CD8^+$ T cell responses in addition to directly participating in the killing of virally infected cells via Fas/Fas-L mediated apoptosis and perforin/granzyme B dependent pathways [221-223]. In contrast, influenza virus infection has been shown to inhibit the development and recruitment of Th2 cells into the lungs (IFN-γ dependent) [224]. In addition to their more well characterized role in controlling the numbers and activities of diverse cell types [225], $CD4^+$ regulatory T cells have been shown to play a critical role in tissue repair and following IAV infection [226], while also promoting the differentiation of Tfh cells [227].

Upon primary IAV infection, $CD8^+$ T cells are activated by APCs (e.g., macrophages and dendritic cells) in the lymphoid tissues, proliferate, and are then recruited to the sites of viral infection through chemokine-chemokine receptor dependent interactions [220, 228]. Once to the site of infection, $CD8^+$ T cells continue to proliferate following interactions with dendritic cells [229]. , and control IAV through the production of antiviral cytokines (e.g., TNFα, MIP-1β and IFNγ), in addition to lysis of virally infected cells through the release of perforin and granzyme, the Fas-FasL pathway, and the TNF-related apoptosis inducing ligand (TRAIL) pathway [230-232]. $CD8^+$ T cells play a critical, but non-essential role in the control of influenza virus infection. In their absence, control of viral replication can still occur through antibody-

dependent mechanisms, but this is a slower less efficient process [170]. $CD8^+$ T cells generally recognize internal more conserved viral epitopes (e.g., M1, NP, and polymerase proteins) and recent work supports a greater cross-reactive potential of $CD8^+$ T cells relative to humoral responses [170, 223]. Following control of viral replication, most $CD8^+$ T cells undergo apoptosis, while a few will go on to form a pool of memory cells [223].

Antibody production by B cells is the primary mechanism for the prevention of IAV reinfection, but in the absence of a $CD8^+$ T cell response, the humoral immune response has been shown to be sufficient to control primary IAV infection, albeit at a slower rate [170, 233, 234]. The antibody responses to IAV primarily targets the HA and NA proteins, and the presence of antibodies recognizing these two proteins being correlating with protective immunity [220], while antibodies against M2 and NP have also being demonstrated [235]. HA-specific antibodies can inhibit viral attachment and entry and are thus capable of neutralizing viral activities [170, 220, 233, 234]. In addition to viral neutralization, humoral immune responses also contribute to the control of IAV infection though antibody-dependent cellular cytotoxicity, antibody-induce inflammation, and antibody-dependent cellular phagocytosis dependent mechanisms [197, 233, 234, 236-238]. Virus-specific IgG, IgM, and IgA antibodies have all been correlated with the resolution of primary IAV infection, or protection against secondary challenge [239-241].

Animal models of influenza A viruses

In order to expand our understanding of IAV immunology, pathogenesis, and host contributions to disease severity, several animal models of influenza virus infection have been developed, with mice being the most widely used [242, 243]. The preferential use of mice in the study of IAV, is in part due to their small size, low cost, ease of animal husbandry, and the wide availability of mouse-specific reagents [242, 243]. However, most human influenza strains do

not replicate efficiently in mice and require adaptation to achieve adequate viral replication, with A/Puerto Rico/8/1934 (H1N1; PR8), mouse adapted A/California/O4/09 (H1N1; ma2009), and A/WSN/33 (H1N1; WSN) being examples of commonly used mouse-adapted strains [180, 242-244]. Disease following IAV infection in mice typically presents with hypothermia, weight loss, hunching, lethargy, ruffled fur, and dyspnea, while IAV transmission within mice does not occur primarily due to the preferential involvement of the deeper lungs [180, 242-245]. Furthermore, within mice, differences in genetic background across murine strains can influence the outcomes of infection. For example, it was recently shown that inconsistencies in reported alveolar macrophage depletion following IAV infection could be explained by mouse strain-dependent differential responses, with decreased numbers of AM being seen in BALB/c mice, but not in C57BL/6 mice [246]. Moreover, the volume of inoculum in which a given infectious dose of IAV is given can influence infection outcome making confounding comparisons across studies [247].

Ferrets are another commonly used model and are naturally susceptible to most human influenza strains without the need for prior viral adaptation in addition to being supportive of intra-species transmission [242, 248-250]. Furthermore, ferrets are considered the best model for recreating human disease, and like humans, infection with seasonal IAV strains primarily causes upper respiratory tract infections in contrast with the lower-respiratory tract infections seen in mice [242, 248-250]. However, their size, expense, handling requirements, and reduced reagent availability, has limited their use [248]. Guinea pigs can support replication of human viral isolates without prior adaption and can sustain IAV transmission, but disease following infection in guinea pigs is clinically unapparent [242, 243, 245, 251]. Syrian hamsters have also been evaluated as a model of influenza, but as with guinea pigs, while they are susceptible to infection to infection without viral adaptation, disease is clinically unapparent [252-254].

Nonhuman primates have also been used in IAV research due to their genetic and physiological similarities with humans, but their use is rare due to their high cost and limited availability [242, 243, 255].

Influenza A virus pathogenesis

Infection with IAV generally results in mild to moderate infections of the upper respiratory tract characterized by the rapid onset of symptoms that often including fever/chills, sore throat, fatigue, nasal congestion, dry cough, rhinorrhea, headache, anorexia and malaise [256-259]. Gastrointestinal symptoms are also frequently reported including nausea, abdominal pain, vomiting, and diarrhea [257, 260]. In some instances (i.e., with severe infection or following infection with highly virulent strains), infection can spread to the lower respiratory tract with disease progressing to include both viral and bacterial pneumonia, edema, alveolar hemorrhaging, acute respiratory failure, and death [261-263]. Extra respiratory complications have also been reported, and include cardiac insufficiency, conjunctivitis, neurological involvement (e.g. Guillain-Barre syndrome and Reyes syndrome), and liver disfunction [263, 264]. Furthermore, age and the presence of comorbidities, have been shown to alter the clinical presentation of IAV infection (e.g. reduced fever in the elderly) [265, 266].

Disease following IAV infection is largely immune mediated with many of the same immune components needed to control the viral infection contributing to disease when excessive or improperly regulated [228, 267, 268]. Infection of epithelial cells and subsequent innate immune cell activation can result in the massive release of proinflammatory cytokines (i.e., interferons, IL-1β, IL-6, IL-8 and TNFα) and chemokines (i.e., CCL3 and CXCL1) [269-271]. If improperly regulated, this cytokine storm can lead to excessive cellular infiltration, pulmonary inflammation, tissue damage and loss of function [269-271]. In addition to the magnitude of the

cytokine and chemokine response, aberration in the timing and location of these responses can contribute to disease and loss of tissue function [269]. Differences in the numbers and kinetics of immune cell influx into the lungs during infection can also greatly impact IAV pathogenesis [272-274]. For example, $CD8^+$ T cells play a critical role in controlling viral replication by direct killing of virally infected cells and the production of antiviral cytokines including TNFα and IFNγ [231, 275, 276]. Conversely, IFNγ production by $CD8^+$ T cells is associated with impaired lung function [232] and $CD8^+$ T cells have been shown to damage uninfected epithelial cells and reduce barrier function through TNFα and IFNγ production [277]. Protection from severe disease with influenza requires a balance between mounting an adequate immune response to control IAV infection, while subsequently promoting is contraction [267, 278], but mechanisms regulating these processes are only partially known.

Risk factors for severe influenza

Not all groups are at equal risk for severe or complicated disease following IAV infection, with several biological host factors being identified to contribute to increased disease severity. Notably, these include advanced age, sex, obesity, pregnancy, chronic immunosuppression, and the presence of comorbidities such as emphysema and cardiovascular disease [262, 279-285]. Females generally mount more robust immune responses to both infection and vaccination than males with greater cell-mediated and humoral immunity often being reported in both humans and in animal models [56, 143, 145, 286]. Conversely, these differences in immune response can also lead to enhanced immunopathology, and in the case IAV, worsen infection outcomes [145, 148, 286-289]. In women of reproductive ages, the rates of clinical infection, hospitalization, and mortality have been all observed to be higher in females of reproductive ages relative to age matched males [144, 290-293]. In contrast, during the 1918 H1N1 influenza pandemic, infection

was disproportionately fatal in young adult males (i.e., 20-40 years of age) [294, 295]. In murine models, most, but not all, studies shown that infection with either H1N1, H3N2, or H7N9 viruses results in greater pulmonary immune activation and increased disease severity in female relative to male mice [164, 287, 296-301]. For example, pulmonary concentrations of proinflammatory cytokines (e.g., TNF-α, IFN-γ, IL-6, and IL-12) and chemokines (e.g. CCL2, CCL5, and CCL12) are greater during IAV infection in females than males [164, 287]. Moreover, following IAV infection, males also repair damaged pulmonary tissue faster than females at least in part through increased expression of the epidermal growth factor amphiregulin [302]. Though of potential therapeutic significance, the mechanisms underlying the differences between males and females are unclear, but differences in sex steroid signaling (i.e. androgens), sex chromosome complement, and epigenetic regulation, have been proposed [145, 288, 303].

Disease burden and cost of influenza are disproportionally highest among individuals over the age 65. Despite higher infection rates in infants and children [304], individuals over the age of 65 account for 54 to 70% of all influenza related hospitalizations and 70 to 90% of all influenza related deaths in the United States [181, 305], while among individuals 65 years or older, males are more likely than females to be hospitalized and succumb to seasonal influenza [291, 306]. In China, older males have been shown to 2.4 times more likely to die following exposure to H7N9 than their female counterparts [307]. In Australia, a male bias has been reported in the relative rate of those seeking medical care for both seasonal and 2009 pandemic H1N1 IAV infection in elderly age groups despite a female bias in reproductive age cohorts [293]. Though few murine studies have used male mice, aged mice have altered pulmonary inflammatory responses, reduced numbers and activity of virus-specific $CD4^+$ and $CD8^+$ T cells during virus clearance, lower antibody responses to live virus and vaccines, and greater disease severity than their young adult counterparts [273, 308, 309]. Whether age-related changes in

sex hormone concentration contribute to the male bias in IAV severity has not been adequately explored.

Recently obesity was recognized as an independent risk factor for severe complications following IAV infection after it was associated with an increased risk of hospitalization and death during the 2009 H1N1 IAV pandemic [310-313]. These clinical observations have since been recreated in murine models of obesity, where obese mice, whether diet or genetically induced, experience increased clinical severity, increased lung damage, and increased mortality relative to non-obese controls [314, 315]. In studies of both mice and humans, obesity is associated with higher levels of proinflammatory cytokines and chemokines (e.g., IL-1β, IL-6, CXCL2 and CCL2), dysregulation of $CD4^+$ and $CD8^+$ T cell responses (e.g., decreased IFN-γ, granzyme B, and IL-12r expression), and the impaired ability to resolve pulmonary damage following IAV infection [316-318]. Furthermore, in both mice and humans, obesity is associated impaired antibody and memory $CD8^+$ T cell responses to IAV vaccination [319, 320]. Notably, obesity in males has been associated with reduced serum testosterone concentration, but implications of this on IAV pathogenesis are unclear [321-323].

Taken together, these data suggest that in males, low testosterone may be associated with increased severity of IAV infection. Given its well described immunoregulatory role, I hypothesize that testosterone reduces the severity of IAV infection by controlling inflammation and regulating the immune response to infection.

Malaria Vaccination

Introduction to malaria

Malaria is caused by single-celled *Plasmodium* parasites belonging to the phylum Apicomplexa [324]. These parasites exist in complex life-cycles alternating being infection of an

invertebrate vector and infection vertebrate hosts including reptiles, birds, and mammals [325]. Over 200 species of *Plasmodium* have been described with each species exhibiting relatively high host vector and vertebrate host specificity [325, 326]. Infection in humans is primarily caused by four species of malaria: *P. falciparum, P. malariae, P. ovale, and P. viv*ax [324]. Zoonotic spillover of a 5th species, *P. knowlesi*, has been observed in Southeast Asia, and can cause severe and sometimes fatal infections in humans [327]. Humans become infected with malaria following sporozoite inoculation during female *Anopheline* mosquito blood feeding [324, 328]. Following inoculation, *Plasmodium* sporozoites travel through the bloodstream to the liver and establish the hepatic cycle (i.e., pre-erythrocytic stage) of malaria infection [324, 328, 329]. In this stage, sporozoites invade hepatocytes, maturates into schizonts, and releases thousands of merozoites into the bloodstream following maturation [324, 328]. Once in the blood stream, the merozoites go on to infect red blood cells establishing the erythrocytic stage of infection [324, 328]. The erythrocytic stage of malaria infection is characterized by episodic cycles (i.e., every 2-3 days) of red blood cell invasion, merozoite replication, and lytic release from the infection red blood cells [324, 328-330]. A portion of merozoites, for reasons that are not entirely clear, will go on to develop into gametocytes which are the sexual stage of *Plasmodium* lifecycle and the stage infectious to the *Anopheline* mosquito [324, 328]. Following mosquito infection during blood feeding, the gametocytes undergo sexual reproduction, and reestablish salivary gland infection as sporozoites after several rounds of replication and maturation [324, 328]. Each stage of this life-cycle is characterized by complex biological differences in parasite physiology that carry unique consequences for the host, and significant variation between malaria strains occur [324, 328-330].

Despite a 30% decline in clinical disease and 47% decline in mortality between 2000 and 2013, malaria continues to represent a significant global burden of disease [331]. In 2017,

malaria was estimated to result in 216 million clinical cases and 445,000 deaths [332]. This was mostly in infants and young children, and worldwide, malaria accounts for approximately 5% of all under 5 child mortality [333]. Of fatal cases, more than 90% occur in sub-Saharan Africa and are attributable to infection with *P. falciparum* [328]. The disease presentation of malaria is complex and varies by the malaria species and age of the patient involved [334]. The hepatic stage is largely asymptomatic, while symptomatic disease primarily occurs as a result of lytic parasite release during blood stage infection [324, 328]. Uncomplicated disease in humans ranges from asymptomatic infection to non-specific moderate disease characterized by fever, chills, headache, profuse sweating, diarrhea, jaundice vomiting and anemia [324, 328, 330], with the onset of symptoms appearing 10 to 15 days following infection [330]. Severe malarial infections are often attributed to *P. falciparum* infection with and can result in severe anemia, cerebral involvement, coma, pulmonary complications, hyperglycemia, and acute kidney failure and death [330, 334]. Furthermore, malaria in pregnancy can involve parasitic invasion of the placenta leading to poor outcome for both the fetus and mother [335].

In response to this high public health burden, the World Health Organization has set out a strategy to achieve a 90% reduction in malaria cases and mortality by the year 2030 [336]. Current methods to interrupt malaria transmission include the early diagnosis and treatment of cases, vector control (e.g. insecticide-treated bed net use and residual indoor insecticide spraying), prophylactic drug treatment, and healthcare capacity improvements [337]. However, these interventions are economically costly and are unlikely to achieve these goals on their own in the presence of increasing antimalarial drug and insecticide resistance [337-341]. To complement the above listed approaches to malaria control, the need for a malaria vaccine that is at least 75% protective has been identified by the World Health Organization [342].

Malarial Vaccines

Despite the high development costs to date, an effective vaccine and implementation strategy would likely be the most cost-effective method for controlling malaria [328, 337]. However, given the extraordinary complexity of the malaria parasite and it's life-cycle, thus far malaria vaccine development has been largely unsuccessful [328, 337]. Due to its high contribution to global disease, *P. falciparum* has been the primary target vaccine develop, while vaccines targeting *P. vivax* are also in development [331]. As a result of the complexities of the malaria life-cycle and the resulting changes in *Plasmodium* antigen expression varies by developmental stage, and vaccines can be broadly classified into categories based on the life-cycle stage that target [331, 343, 344]. Transmission blocking vaccines target the sexually reproductive gametocyte stage of malaria with aim of blocking the establishment of infection in the mosquito [345, 346]. In this potential vaccine strategy, the result of immunity generated through vaccination, is not a reduction in disease in the recipient, but rather the interruption of transmission within a vaccinated population [345, 346]. Other vaccines in early stage development, include those that target the blood, or erythrocytic, stage of infection which aim to reduce clinical disease associated with malaria infection, by target merozoite protein expression [331, 343, 347, 348].

The most successful candidate vaccines to date, target the pre-erythrocytic stage (i.e. sporozoite stage) of malarial infection with the goal of blocking hepatic invasion and subsequent progression to blood stage infection [331, 344, 349]. However, with this strategy, sterile 100% protective immunity is required to prevent parasite escape and life-cycle progression [331]. Currently vaccines are in development based on both whole sporozoite vaccine models (e.g., intravenous inoculation with radiation attenuated or genetically attenuated sporozoites) as well peptide-based vaccines that primarily targeting the circumsporozoite protein (CSP), which is the

immunodominant antigen expressed by sporozoite stages. [331, 344, 349]. Including in this category is the RTS,S/AS01 vaccine which is the leading candidate malaria vaccine and is currently undergoing pilot implementation in several regions of sub-Saharan Africa [342]. The RTS,S/AS01 vaccine consists of the carboxy-terminal region of the P. falciparum CSP surface protein along with the hepatitis B surface antigen. In Phase III clinical trials, the RTS,S/AS01 vaccine was shown to be moderately efficacious with 18-36% protection against clinical disease in young boys and girls (i.e., 6-12 weeks and 5-17 months) [350]. Though protection induced by the RTS,S/AS01 vaccine is far lower than 75% vaccine efficacy target, it represents a substantial improvement over previous vaccine designs [342].

Animal models for malaria research

As with influenza, mice are the primary animal model for malaria research and this preference is driven by their small size, low cost, ease of animal husbandry, and the wide availability of mouse-specific reagent reagents [326, 351, 352]. However, the relative high host specificity of *Plasmodium* species, prohibits their use in the study of malaria species that cause disease in humans and limits their use for pre-clinical research [326, 351, 352]. Instead rodent specific *Plasmodium* species are commonly used including *P. berghei, P. yoelii, and P. chabaudi* [326]. Though disease is often similar, substantial genetic and physiological differences between rodent and human species of malaria can make interpretation and translation of findings difficult [353]. Furthermore, not all aspects of infection with human species of malaria can be modeled in traditional rodent models [326, 354]. To circumvent some of these limitations, and facilitate the study of human species of malaria (i.e., *P. falciparum*) in a small animal models, both transgenic and humanized mice that contain human genes or tissues have developed [326, 354]. For example, humanized mice containing human liver cells or transplanted tissues can be

used to study *P. falciparum* pre-erythrocytic stage infection [326, 355]. To facilitate pre-clinical vaccine study and evaluate antigenicity of potential target antigens, transgenic parasites have been also been developed that allow for study of human malaria parasites in mice [356]. As an example, for all studies presented in Chapter 4 of this dissertation, transgenic *P. berghei* parasites expressing a *P. falciparum* CSP construct were used [357].

Despite limitations including ethical concerns, large size, high cost, and outbred genetic background which limits reproducibility, nonhuman primates (NHP) play a valuable role in malaria research [326, 355]. The primary advantages conveyed using NHPs is their susceptibility to human malaria species, similar disease presentation, and physiological similarities to humans [326, 355]. These characteristics make them ideal pre-clinical models for vaccine trials, safety and efficacy evaluation of treatments and interventions, and for pathogenesis studies. In NHP species not supportive of infection with human malarial species, research is facilitated by the use of *P. cynomolgi*, which is more closely related to human malarial parasites than those that infect rodents [326, 355].

The effects of sex and sex-hormones on malaria infection and vaccination

Among humans, both the incidence and intensity (i.e. parasite density) of *Plasmodium* infection is often greater in men than in women, with both adult and juvenile males being more likely than females to be asymptomatically infected [147, 358-365]. However, despite the male bias in infection frequency and intensity, malaria is often more severe in both juvenile and adult females, with the frequency of hospitalization, sepsis, anemia, shock, and mortality being more common in female patients [148, 362, 366-373]. Furthermore, women have been observed to be at a greater risk of death from acute respiratory distress syndrome (ARDS) following *P. vivax* infection relative to men, while pregnancy has been identified as an independent risk factor for

both the development of severe malaria and for mortality following the diagnosis of cerebral malaria [374-376]. Though gender differences in societal roles and care seeking behaviors are likely to contribute to some of the observed differences in the outcomes of malaria infection [377, 378], observations that both *Plasmodium* parasite density as well the frequency of malaria positive blood smears increases in males relative to females with the onset of puberty suggests that biological factors such as testosterone levels are likely to be involved [146, 360] . Consistent with this idea, in adult males, serum testosterone concentrations have been positively correlated with *P. vivax* parasite load, suggesting that testosterone may inhibit resistance to malaria infection [379].

As with humans, sex differences in the outcomes of infection have also been described in murine models of malaria. However, in contrast with observations in humans, the severity of infection with both *P. chabaudi* and *P. berghei* is often greater in male than in female mice, with males generally experiencing greater mortality and being slower to recover from infection induced anemia, body mass loss, and hypothermia [380-382]. Moreover, infection in female mice results in reduced parasitemia and a more robust immune response to infection (e.g. increased IFNγ, IL-10, TNFα and plasmodium-specific IgG1 and IgG2b antibody production) relative to males [380, 382, 383], suggesting that females may be more resistant to infection. In *P. berghei* models of cerebral malaria, increased evidence of oxidative stress in the brain (e.g., increased nitric oxide concentrations), and disruption of neurotransmitters (i.e., dopamine, epinephrine, and serotonin) is seen in males relative to females [382]. Further, more pronounced disruption of the urogenital tract has been described in male mice infected with *P. chabaudi* [384].

In mice, testosterone is associated with reduced resistance to *P. chabaudi* and *P. berghei* blood stage infection in both males and females [381, 385-389]. Castration of male mice

reduces, while subsequent testosterone treatment increases, mortality and worsens blood-stage infection severity [386, 390]. This increased survival in testosterone-depleted males is associated with increased total leukocyte numbers and the greater proliferative potential of B and T cell populations following *Plasmodium* infection, while NK cell populations are reduced in these same mice [383, 386, 389]. In female mice, testosterone treatment also increases mortality following *P. chabaudi* infection, with these effects persisting following withdrawal of testosterone treatment while being associated with downregulation of hepatic proinflammatory genes (e.g., *Ifnγ* and *Igk-C*) [390, 391]. Consistent with estrogen playing a protective role, gonadectomy of female mice (i.e., depletion of estrogen), reduces survival following *Plasmodium* infection relative to intact females [385, 390]. In contrast, others have reported that estrogen treatment of both male and female C57BL/10 mice inhibits the acquisition of immunity to *P. chabaudi* by naïve mice, but does not reduce pre-existing immunity [392].

Sex differences in vaccine-induced immune responses are well documented for vaccines that protect against viruses and bacteria [57, 166], but the implications of sex differences in the response to potential vaccines targeting parasitic infections, including candidate vaccines against malaria are rarely considered. In Phase I immunogenicity trials of the malaria candidate vaccine SPf66, a synthetic peptide-based vaccine targeting the blood stage of *P. falciparum*, adult women (n = 4) were observed to mount greater humoral responses and PBMC lymphoproliferation followed peptides stimulation than men [348]. Consistent, with observations that female mice are more resistant to *Plasmodium* infection, females have been shown to be better protected against challenge than males following vaccination with the surface membranes of *P. chabaudi*-infected red blood cells [168]. To date, no pre-clinical study has been adequately designed or analyzed to study sex differences in the immunogenicity and efficacy of pre-erythrocytic stage malaria vaccination. Additionally, the RTS,S/ASO1 vaccine has

been associated with higher all-cause mortality in girls, but not in boys, in both age groups in which the vaccine was tested [393]. Whether this sex-dependent vaccine outcome represents a difference in the immune response to RTS,S/ASO1 vaccine is unknown.

Taken together, these data suggest that females may better protected following pre-erythrocytic stage malaria vaccination. Given the well characterized immunosuppressive properties of testosterone, I hypothesize that testosterone in males will suppress both cell-mediated and humoral immune responses to vaccination and reduce resistance to sporozoite challenge.

Specific Aim 1

The severity of influenza increases with age, with hospitalization and death rates generally being higher in older males than in age-matched females. In males, circulating testosterone levels also decline with age, and I hypothesized that reduced testosterone concentrations in aged males contribute to age associated increases in influenza severity. To assess the role of testosterone in the severity of IAV infection, I first used a model where, young adult and aged male mice were left intact and infected with a low dose of IAV. As testosterone can reduce inflammatory responses, I hypothesized that higher testosterone concentrations in young males would specifically lead to reduced pulmonary inflammation.

To reduce the influence of age-related factors other than testosterone on the severity of IAV, I next surgically reduced testosterone in young adult male mice and exogenously replaced testosterone in aged male mice. To date, no studies have assessed the effects of testosterone replacement on infection in aged males. I hypothesized that the depletion of testosterone in young males would increase, while the replacement of testosterone in age males would reduce the severity of IAV infection.

Specific Aim 2

My findings from Specific Aim 1 suggested that low testosterone, whether age-related or surgically induced in young males, increased the severity of influenza virus infection. These effects were most pronounced during the recovery phase of infection (i.e., after virus had been cleared), and were associated with the delayed resolution of pulmonary inflammation in young males, independent of changes in viral replication. As few studies have directly evaluated the effects of testosterone on the immune response to viral infection, I wanted to further evaluate whether the delayed resolution of pulmonary inflammation was associated with the changes in the immune response to the virus. I hypothesized that testosterone was reducing the severity of IAV infection by accelerating the contraction of the immune response following control of viral replication. Given that $CD8^+$ T cells are major contributors of both viral clearance and immune-mediated pathology, I further hypothesized that testosterone may be acting to promote the resolution of $CD8^+$ T cell responses following viral clearance.

In this Specific Aim 2, I elected to evaluate the effects of testosterone on the immune response to IAV using young adult male mice to eliminate other age-related changes that may be occurring. For these studies, I also used the androgen receptor antagonist flutamide and non-aromatizable dihydrotestosterone to confirm that the effects of testosterone were mediating through androgen receptor signaling and not through estrogen receptor signaling. I hypothesize that the protective effects of testosterone on IAV pathogenesis are mediated through direct androgen receptor signaling in virus-specific $CD8^+$ T cells.

Specific Aim 3

Adult females tend to develop greater adaptive immune responses than males following receipt of either viral or bacterial vaccines, in both preclinical animal studies and human clinical trials. While there is currently no approved malaria vaccine, several anti-sporozoite vaccines, are in development. Few studies have considered the influence of sex and sex hormones on the efficacy of vaccines targeting parasitic infections including malaria. Consistent with data from viral and bacterial vaccines, I hypothesized that females would mount greater immune response and be better protected following challenge than males. To date, no studies have assessed the effects of testosterone on the efficacy of pre-erythrocytic stage malaria vaccination. Given that, testosterone was observed to be immunosuppressive in Specific Aims 1 and 2, I further hypothesized that testosterone would suppress adaptive immune responses and protection following vaccination.

In this Specific Aim 3, I used a well-established whole sporozoite vaccine model, where both adult or juvenile male and female mice were vaccinated twice with irradiated transgenic *P. berghei* sporozoites expressing the *P. falciparum* CSP protein. Forty-five following the second inoculation, mice were challenged with transgenic *P. berghe*i via mosquito bite or intradermal challenge. For these studies, I also elected to study the influence of male and female sex hormones on sex-specific differences infection through surgical castration. I hypothesize that the depletion of female specific sex hormones would reduce, while the depletion of testosterone in males would increase, vaccine efficacy.

Chapter 2

Age and Testosterone Mediate Influenza Pathogenesis in Male Mice

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Abstract

Influenza severity increases with age, with hospitalization and mortality rates during seasonal influenza epidemics being higher in older men than age-matched women. Based on knowledge that with age, circulating testosterone levels decline in males, we hypothesized that reduced testosterone contributes to age-associated increases in influenza severity. A murine model was used to test this hypothesis. As in men, testosterone concentrations were lower in aged (18 months) than young (2 months) male C57BL/6 mice. Following inoculation with influenza A virus (IAV), aged males experienced greater morbidity, clinical disease, and pulmonary inflammation than young males, and had lower neutralizing and total anti-influenza IgG antibody responses. Peak titers of virus in the lungs did not differ between aged and young males, but virus clearance was delayed in aged males. In young males, removal of the gonads increased, whereas treatment of gonadectomized males with testosterone reduced, morbidity, clinical illness, and pulmonary pathology, but did not alter viral replication. Treatment of aged males with testosterone improved survival following infection but did not alter either virus replication or pulmonary pathology. These results indicate that low concentrations of testosterone, whether induced surgically in young males or naturally occurring in aged males, negatively impact the outcome of influenza.

Introduction

Every year in the United States, the disease burden and cost of influenza is disproportionately highest among individuals over age 65. Ninety percent of deaths from seasonal influenza are in people over 65 years of age [394]. Although it is well established that immune protection declines in aged individuals, sex-based differences in immunosenescence are not often considered [395]. It has been reported that among unvaccinated individuals 65 years or older, males are more likely than females to be hospitalized and succumb to seasonal influenza [291, 306]. During the H7N9 outbreak in China, older males were 2.4 times more likely to die following exposure to H7N9 than their female counterparts [307]. In aged individuals who receive either the standard or high dose trivalent inactivated influenza vaccine, antibody titers are 2 to 3-times lower in males than females [395, 396]. The mechanisms that mediate reduced protection against influenza in aged males have not been reported.

As in humans, murine studies suggest that dysregulated immune function in aged individuals underlies severe outcome from influenza A virus (IAV) infection. In response to IAVs, aged mice have elevated pulmonary inflammatory responses [273], reduced numbers and activity of virus-specific CD8+ T cells during virus clearance [308], and lower antibody responses to live virus and vaccines [309] than their young adult counterparts. An informal analysis of the literature revealed that, as in humans, most murine studies on age-associated changes in IAV pathogenesis and vaccination either do not report the sex of the mice (~50%) or use female mice only (~35%). A minority use either mixed sexes or male mice, which follows general published trends in the fields of immunology and infectious diseases [397].

In men, there is a gradual decline in testosterone secretion that begins after age 30, with the signs and symptoms of testosterone deficiency typically presenting after age 65 [398]. Age-related reductions in testosterone production are associated with symptoms including

decreased libido, erectile dysfunction, fatigue, depression, reduced strength, bone loss, and increased abdominal fat [31]. Although concerns about the effects of testosterone replacement on cardiovascular disease have been raised [399], most studies suggest that testosterone replacement in aged males with hypogonadism results in health and quality of life benefits [31, 399, 400]. To date, there are no studies that have considered the immunological consequences of testosterone replacement in aged males. We hypothesize herein that reduced testosterone concentrations are detrimental for susceptibility to and the outcome of influenza, and that susceptibility would be reduced, and the outcome improved by testosterone replacement. With the knowledge that testosterone can signal through androgen receptors in immune cells to regulate responses to immunological stimuli, including viruses and vaccines [104, 148], and that exogenous treatment of young adult male mice with testosterone generally reduces the synthesis of proinflammatory cytokines (e.g. IFN-γ and TNF-α), increases anti-inflammatory cytokines (e.g. IL-10), and reduces helper T cell type 1 (Th1) activity [23], we further hypothesize that elevating serum testosterone levels in both hypogonadal young and aged males would improve the outcome of influenza by mitigating inflammation.

Materials and Methods

Animals

Young (10-12 weeks of age) and aged (17-18 months of age) adult male C57BL/6 mice were obtained from Charles River or the National Institute of Aging, respectively; and housed 3 to 5 per microisolator cage under standard BSL-2 housing conditions, with food and water *ad libitum*. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Experiments were conducted as a series of replicates and animal numbers are provided in the legends.

Gonadectomy, Testosterone Administration and Quantification

Young adult male mice were anesthetized with ketamine/xylazine cocktail and bilaterally gonadectomized as previously described [164, 298]. All animals were given two weeks to recover from surgery prior to testosterone treatment. Testosterone was administered by subcutaneously implanting a silicone capsule (0.040 in inner diameter, 0.085 in outer diameter, 7.5 mm length) containing 100% crystalline testosterone propionate (Sigma) between the scapulae. The capsules were equilibrated in sterile physiological saline for 12 h at 37^{o}C prior to implantation. Animals in the placebo group received implants of empty capsules. Testosterone concentrations in serum were measured by radioimmunoassay using antibodies from Fitzgerald (Acton, MA) and tracer testosterone ([1,2,6,7-3H(N)]-Testosterone) from PerkinElmer (Waltham, MA) or EIA (Immuno-Biological Laboratories, Inc.) following a standard steroid extraction. All males were treated with testosterone for one week prior to infection.

Virus Infection and Quantification

The mouse-adapted H1N1 influenza A viruses (IAVs), A/Puerto Rico/8/34 (PR8: H1N1; courtesy of

Dr. Maryna C. Eichelberger) or A/California/4/09/H1N1 (ma2009; H1N1; generated by Dr. Andrew Pekosz using a published sequence [401]) were used in all experiments. Mice were anesthetized and then intranasally inoculated with 30μl of DMEM for the mock-infection or maH1N1 diluted in DMEM (PR8 = 0.05 MLD_{50}; ma2009= 0.1 MLD_{50}). For virus quantification, log_{10} dilutions of lung homogenates were plated onto a monolayer of Madin-Darby canine kidney (MDCK) cells in replicates of 6 for five days at 32°C. Cells were stained with naphthol blue black (Sigma Aldrich) and scored for cytopathic effects (CPE). The 50% tissue culture infectious dose ($TCID_{50}$) was calculated according to the Reed-Muench method and was used to back titer all inoculums.

Sample Collection

Body mass and rectal temperature were recorded daily for 21 days and clinical disease scores were recorded at selected time points during the morbidity studies. Clinical disease scores for IAV infected mice were based on five points, with one point given for each of the following: dyspnea, piloerection, hunched posture, absent escape response, and a fifth point given if deceased [402]. For terminal studies, males were euthanized at designated days post-inoculation (dpi), at which time serum and whole lungs were collected.

Lung Inflation and Histopathology

Lungs were inflated at constant pressure and then fixed for 48 h with buffered zinc formalin fixative (Z-Fix, Anatech). Lungs were embedded in paraffin, cut into 5μm sections, and mounted on glass slides. Consecutive tissue sections were stained with hematoxylin and eosin for histopathological scoring. Tissue sections were evaluated for vascular, bronchiolar or alveolar inflammation and edema, and were assigned a score on a 0-3 scale (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, and 3 = severe inflammation). The cumulative

inflammation score represented the sum of each individual inflammation parameter. The percentage of affected lung area within each section was calculated from binary images created using ImageJ software (NIH), and values represent the average of three random 10X fields within each tissue section [403]. Scoring was performed by a single blinded observer in consultation with a boarded veterinary pathologist. Representative images were taken using a Nikon Eclipse E400 at 20x magnification.

Antibody Neutralization Assay

Serum samples were added to serum-free infection media and serially diluted (1:2 dilutions). One hundred $TCID_{50}$ units of virus were added to the diluted samples and incubated at room temperature for 1 h. The diluted samples and virus were added to MDCK cells at 100% confluence in 96 well plates and incubated overnight at 37^{o}C. After 16-18 h of incubation, the inoculums were removed, the cells were washed with PBS plus calcium and magnesium, fresh infection media was added, and the cells were incubated for 5 days at 32^{o}C. Each sample dilution series was run in quadruplicate and the titer was calculated as the highest serum dilution that eliminated virus CPE in 2 out of 4 wells per dilution.

Anti-influenza total IgG ELISA

ELISA plates (Microlon 96 well high binding plates; Greiner Bio-One) were coated overnight at 4^{o}C with 100 ng of purified PR8 or ma2009 virus, after which plates were washed and blocked for 1 h with blocking solution (10% dry skim milk powder in PBS). Plates were washed, duplicate diluted serum samples were added in a 2-fold series starting at 1:1000, and plates were incubated at 37^{o}C for 1 h. Anti-mouse IgG secondary antibody (1:5000; Peroxidase AffiniPure goat anti-mouse IgG; Jackson Immunoresearch Laboratories) was added and plates were

incubated for 1 h at 37^{o}C. Reactions were developed with 3,3',5,5' tetramethylbenzidine (TMB; BD Biosciences) and stopped using 1N HCL. Plates were read at 450 nm absorbance on a plate reader. To determine the antibody titer, a cutoff value was determined by multiplying the average ELISA values of serum from naïve animals at each dilution by 3. The sample ELISA titer was the highest serum dilution of that sample series with a value above the cutoff.

Statistical Analyses

Morbidity and clinical data were analyzed with a MANOVA with one within-subjects variable (days) and one between-subjects variable (treatment), and significant interactions were further analyzed using planned comparisons. Antibody responses, virus titers, and histopathological data were analyzed using two-way ANOVAs or t-tests, and significant interactions were further analyzed using the Tukey method for pairwise multiple comparisons. Mean differences were considered statistically significant if $p<0.05$.

Results

Influenza virus infection is more severe in aged compared with young males

Circulating testosterone concentrations were 3-fold higher in uninfected young compared with aged adult male C57BL/6 mice (**Fig 2.1A**; $p<0.05$), which are within the ranges reported for inbred laboratory strains of mice [404-407]. Testosterone concentrations were also measured at 3, 7, 14, and 21dpi in young and aged male mice infected with a low dose of ma2009 (i.e., a dose previously determined to be sublethal in young male mice) and revealed that in young males, testosterone levels dropped during the acute phase of infection (7 dpi), but then rebounded during the recovery phase of infection resulting in testosterone concentrations that were higher than aged males at 0,3 and 21 dpi only (**Fig 2.1B;** $p<0.05$). In contrast, testosterone levels remained low throughout the course if IAV infection in aged male mice.

To test the hypothesis that persistently low testosterone levels were associated with a worse outcome of influenza in older than younger males, mice were inoculated with a low dose of ma2009 virus and body mass, rectal temperature, and clinical disease were monitored for 21 dpi. Aged male mice experienced greater body mass loss, hypothermia, clinical illness, and mortality than young males (**Fig 2.1C-E** and data not shown; $p<0.05$). Titers of ma2009 were measured at select dpi and although peak viral titers in the lungs did not differ between young and aged male mice, the clearance of infectious virus was significantly delayed in aged males (**Fig 2.1F**; $p<0.05$). In response to infection with a low dose of another IAV, PR8, aged males also experienced greater morbidity and clinical illness than young males (**Fig 2.2A-C**; $p<0.05$), illustrating that the effect of aging on severe outcome from influenza was conserved across historic (PR8; isolated in 1934) and contemporary (ma2009) strains of IAV.

Histological examination of lung tissue collected at several time-points following IAV infection revealed significantly more pulmonary inflammation in the lungs of aged than young

males throughout infection period (**Fig 2.3A**; p<0.05). In affected areas, similar levels of inflammation were seen between young and aged males at 7 dpi (**Fig 2.3B**); however, by 11, 14 and to the greatest extent at 21 dpi, aged males had significantly more pulmonary inflammation than young males (**Fig 2.3B**; p<0.05). When scored by structural regions of the lung, no differences in the distribution of inflammation were observed at 7 dpi (**Fig 2.3C**), whereas perivascular and peribronchiolar inflammation were higher in aged than young male mice at 11 dpi (**Fig 2.3D**; p<0.05). There was a shift in the location of inflammation at 14 and 21 dpi, with increased pulmonary inflammation observed in the terminal bronchioles and alveolar spaces of aged males, predominantly characterized by increased edema at 14 dpi (**Fig 2.3E**; p<0.05) and increased alveolar inflammation and edema at 21 dpi (**Fig 2.3F** and **G**; p<0.05). Given the differences in testosterone concentrations between young and old males, and the rebound in testosterone concentrations seen in young, but not aged males (**Fig 2.1B**) which corresponded with improved recovery from IAV induced pulmonary inflammation (**Fig 2.3B**) during the recovery phase of infection, we hypothesized that testosterone is associated with protection against IAV in young male mice.

Protection against influenza in young males is mediated by testosterone

Previous data illustrate that castration of young male mice prior to lethal IAV infection reduces survival as compared with testis-intact males [298]. To determine whether testosterone directly mediated protection of young males from IAV, young adult male mice were either left intact or castrated to deplete testosterone production and implanted with either placebo or testosterone capsules prior to infection with a low dose of PR8. Castration reduced, and exogenous testosterone treatment of young adult males restored, circulating testosterone levels to within the physiological range of testis-intact young males (**Fig 2.4A**; *p*<0.05). Following

infection with either PR8 **(Fig 2.4A-C)** or ma2009 **(Fig 2.4D-F)**, castration of young adult males increased the severity of IAV, and treatment of castrated males with testosterone at physiological doses reduced the severity of IAV to levels comparable with testis-intact male mice (**Fig 2.4B-E**; $p<0.05$). Neither peak virus titers nor clearance of infectious virus from the lungs were affected by testosterone replacement in young male mice (**Fig 2.4F**).

The lungs of castrated males treated with testosterone showed reduced total inflammation and faster resolution of inflammation than the lungs of placebo-treated castrated males (**Fig 2.5A**). Young castrated males treated with testosterone had less pulmonary pathology at 14 dpi as compared with placebo-treated males, which presented as less perivascular, peribronchiolar, and alveolar inflammation in the lungs (**Fig 2.5B-C**; $p<0.05$). These data illustrate that testosterone is one host factor contributing to protection against influenza in young adult male mice.

Testosterone replacement improves survival from influenza in aged males

To determine if testosterone replacement in aged males could improve the outcome of IAV, aged males were implanted with either placebo or testosterone capsules and circulating testosterone concentrations measured three weeks after implantation. Exogenous testosterone treatment in aged males resulted in circulating testosterone concentrations that were within the physiological range seen in young adult male mice and were significantly higher in the testosterone-treated than in the placebo-treated aged males (**Fig 2.6A**, $p<0.05$). Following infection with a low dose of ma2009, aged males treated with testosterone experienced similar body mass loss and hypothermia as compared with males treated with placebo (**Fig 2.6B** and data not shown). Despite having similar patterns of morbidity, aged males that were treated with exogenous testosterone experienced less clinical disease (**Fig 2.6C**, $p<0.05$) and were

significantly more likely to survive infection than aged males treated with placebo (**Fig 2.6D**, $p<0.05$). Testosterone-treatment did not affect peak virus titers or clearance of infectious virus from the lungs of aged males (**Fig 2.6E**). Replacement of testosterone in aged males also did not signficantly alter pulmonary pathology during the course of infection (**Fig 2.6F**). Taken together, these data suggest that testosterone treatment in aged males is associated with reduced clinical illness and mortality from IAV, but not through reduced virus replication or pulmonary inflammation.

Age rather than testosterone predicts antibody responses to IAVs in males

Antibody production and the ability of antibodies to neutralize IAVs are relative correlates of protection against subsequent IAV infection and for efficacy of influenza vaccines [408], which are reduced in aged individuals. In the present study, aged males produced significantly lower neutralizing and total anti-IAV serum IgG antibodies than young males against both PR8 and ma2009 (**Fig 2.7A-D**; $p< 0.05$ in each case). Treatment of young adult male mice with testosterone had no effect on neutralizing antibody responses or total anti-IAV IgG responses against IAVs (**Fig 2.7E-H**). Testosterone-treatment also did not affect either neutralizing or total anti-IAV IgG antibody responses against IAVs in aged male mice at 21 dpi (**Fig 2.7I-L**). These data illustrate that despite improving the outcome of influenza in both young and aged males, there was no effect of testosterone on antibody production in either young or aged male mice.

Discussion

Like data in female mice [273, 308, 309], aging was associated with worse IAV infection outcomes in male mice. Aged male mice had lower concentrations of testosterone and experienced greater morbidity and mortality following IAV infection compared to young male mice. Changes in body mass were similar between both young and aged mice during the acute phase of infection, with both groups losing approximately 20% body mass by 8 dpi. However, during the recovery phase, while body mass in young males returned to baseline by 16 dpi, body mass in aged males did not return to baseline within the 21-day study period. This was associated with delayed viral clearance, persistence of clinical disease symptoms, and impaired resolution of pulmonary inflammation in aged compared with young males. These data suggest a correlation between low testosterone and severity of influenza in aged males. This was further supported by observations that in young males, there was a transient decline in circulating testosterone concentrations during the acute phase of IAV infection when pulmonary inflammation was high, which was followed by a rebound in testosterone levels after (i.e., 21 dpi) recovery from IAV. The association between severe disease and declining androgen levels has been previously reported for IAV [164], HIV [409], and tapeworm infections [410]. Presumably, inflammation, and more specifically cytokine secretion, can interfere with steroidogenesis, including testosterone production by the testes [411], which during an acute infection (e.g., IAV) is transient, but during a chronic infection (e.g., HIV) can be long-term and lead to infertility.

To determine the role of testosterone in mitigating IAV-associated disease in the absence of other age-related physiologic change, we conducted mechanistic studies in which testosterone concentrations were depleted by castration and replaced with exogenous continuous-release capsules of testosterone that increased testosterone to within the normal

physiological range for young adult male mice. In young males, castration followed by treatment with testosterone resulted in a clinical phenotype like testis-intact young males after infection with IAV, with testosterone reducing morbidity, clinical disease, and pulmonary inflammation. However, in contrast to aged males, by 21 dpi, gonadectomized young males depleted of testosterone returned to near baseline body mass, demonstrated minimal evidence of clinical disease, and resolved pulmonary inflammation to levels comparable to testosterone treated males. The differences between young gonadectomized males and aged males, both of which had low circulating testosterone, suggest that other age-related physiologic changes in addition to reduced testosterone may contribute to age-related susceptibility to influenza.

In aged males, testosterone replacement reduced mortality and clinical severity, but had minimal effects on morbidity and pulmonary inflammation. It is possible that increased mortality of placebo-treated males created a bias towards animals that survived, and this may have masked differences between treatment groups in recovery from pulmonary inflammation at later time points during infection. Alternatively, other age-related physiologic changes may render the aged population more refractory to testosterone's protective effects.

Overall, testosterone improved the outcome of IAV infection in male mice and physiological doses of testosterone were associated with improved outcomes following infection with two different strains of IAV. Although previous studies using a lethal dose of PR8 in young male mice showed a trend for testosterone and the nonaromatizable androgen, dihydrotestosterone, to reduce mortality from influenza virus infection [164], using low doses of both PR8 and ma2009 resulted in more pronounced effects of testosterone on the outcome of infection. The outcome of IAV infection in testosterone-treated young adult castrated males resembled the outcome of infection in testis-intact male mice, suggesting that testosterone is a significant protective factor against influenza.

The biological activity of sex steroids depends on many factors, including the availability of the unbound ligand, receptor expression and distribution, and nuclear translocation and signaling. We hypothesize that testosterone may have more profound effects in young relative to aged male mice due to age-related changes in androgen receptor expression, nuclear translocation, and signaling [412-415]. Sex steroids can only have their biological effects when decoupled from sex hormone binding globulin (SHBG). Studies in humans demonstrate that SHBG levels increase with age and result in decreased bioavailability of circulating testosterone [416, 417]. Whether similar regulatory processes may be limiting the efficacy of testosterone treatment in aged male mice warrants future study. With age, if the availability of free testosterone or the activity of androgen receptors and associated signaling pathways are altered, then increasing the concentrations of testosterone may not be enough to fully reverse the effects of aging on influenza pathogenesis. Further, whether aging in males is associated with changes in the aromatization of androgens into estrogens requires consideration because at least in female mice, estrogens are anti-inflammatory and improve the outcome of IAV [164, 418]. Along these lines, whether the protective effects of testosterone treatment during IAV infection in males are due to signaling through the androgen or estrogen receptor will be investigated in future studies.

In the present study, testosterone-treatment of young males conferred protection from IAV-associated disease by reducing pulmonary inflammation and tissue damage during the later stages of infection. The precise cellular mechanisms that mediate immune modulation following testosterone replacement remain to be determined. In response to other inflammatory diseases, such as experimental autoimmune encephalomyelitis (EAE) in mice, testosterone is associated with expansion of regulatory T cell and Th17 populations and the reduction of Th1 activity [120, 122]. Generally, castrated male mice have higher numbers of CD4+ and CD8+ T

cells than intact males [419]. Castrated male mice also have higher numbers of macrophages and antigen-specific CD8+ T cells following viral infection than testis-intact males [121]. Although testosterone-induced changes in immune function are beneficial to the outcome of EAE and IAV infection, these same immunological changes can be detrimental to other diseases, including sepsis, trauma-hemorrhage, and wound repair, in which treatment with androgens is associated with depression of cell-mediated immune responses necessary for recovery [420-422]. Characterization of the relative contributions various immune cell populations play in mediating the protective effects of testosterone against IAV warrants additional study.

Human studies reveal that higher levels of testosterone are correlated with lower antibody responses to the trivalent inactivated seasonal influenza vaccine (TIV) [104]. In the current studies, manipulation of testosterone did not affect either neutralizing or total anti-IAV IgG titers in either young or aged male mice. Whether this reflects differences in androgen signaling in humans and mice, systemic immunization versus local pulmonary infection, or use of inactive versus live IAVs requires additional consideration. Instead, age was more likely to predict the antibody response to IAV, with young males having significantly higher antibody titers than aged males.

Study of the hormonal effects on immune responses to IAV has focused exclusively on effects in young adults, and predominantly on hormonal effects in young adult females, which demonstrate profound effects on viral pathogenesis and immune responses to vaccination [164, 402, 423, 424]. No studies have evaluated the relative contribution of age-related reductions in sex steroid levels in explaining why influenza pathogenesis and vaccine efficacy are worse in aged males. Whether testosterone also affects susceptibility to pneumonia following secondary bacterial infection, which is often a principle cause of death from influenza [425-427] warrants

further consideration. We now provide systematic evidence of a role for testosterone in mediating IAV pathogenesis in both young and aged males.

Prescription of testosterone replacement therapy has increased as the population of adults age 65 and older continues to grow [398]. To date, clinical studies have focused exclusively on the impact of testosterone on sexual function, mental health, bone health, muscle mass, and metabolic and cardiovascular diseases [398, 428, 429]. These data highlight additional impacts of testosterone on the immune response and suggests that testosterone replacement therapy may have additional benefits in the context of infectious disease.

Acknowledgments

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Figure legends

Figure 2.1: Effects of age on the outcome of ma2009 virus infection. Circulating concentrations of testosterone were measured in aged (17-18 months; n=9) and young (10-12 weeks; n=7) male mice prior to infection (A) as well as over the course of infection (B). Following intranasal inoculation with a low dose of mouse-adapted influenza A virus (ma2009), body mass (C), clinical disease (D), and survival (E) were monitored for 21 days in aged and young adult male mice (n=15-34/age). Pulmonary viral titers were measured at 3, 7, 11, 14- or 21-days post-inoculation (dpi) (F; n=4-8/treatment/time-point). Significant differences between aged and young adult male mice are represented by asterisks ($p < 0.05$).

Figure 2.2: Effects of age on the outcome of PR8 virus infection. Following intranasal inoculation with a low dose of a historic mouse-adapted influenza A virus (PR8), changes in body mass (A), rectal temperature (B), and clinical disease (C) were monitored for 21 days in aged and young adult male mice (n=10/group). Significant differences between aged and young adult male mice are represented by asterisks ($p < 0.05$).

Figure 2.3: Effects of age on pulmonary inflammation following infection with ma2009. Lungs were collected from young and aged males that were mock infected or infected with a low dose of influenza A virus (ma2009) and euthanized at 7, 11, 14, or 21 days post inoculation (dpi) (n=4-7/age/time-point). Lung tissues were sectioned, stained with H&E, and scored for markers of inflammation, including perivascular inflammation, peribronchiolar inflammation, alveolar inflammation, and edema Cumulative inflammation scores (A), and percent affected area (B) are presented. Histology scores for individual inflammatory parameters were compared between young and aged males at each time post-inoculation (C-F). Representative photomicrographs (G)

are shown for lungs collected from young and aged IAV- and mock-infected males at 21 dpi. Significant differences between aged and young adult male mice are represented by asterisk ($p < 0.05$).

Figure 2.4: Effects of exogenous testosterone-treatment on the outcome of IAV infection in young adult male mice. Young adult male mice were left intact or gonadectomized and either treated with placebo (Gdx) or time-release testosterone (Gdx + T) pellets for one week prior to inoculation with influenza A virus (IAV; PR8). Concentrations of testosterone (A) were measured in serum samples collected 21 days post-inoculation (dpi) (n = 6-17/treatment). Following intranasal inoculation with PR8, changes in body mass (B) and clinical disease (C) were monitored for 21 dpi (n = 8-10/group). A separate cohort of young adult male mice was gonadectomized and either treated with placebo (Gdx) or time-release testosterone (Gdx + T) pellets for one week prior to inoculation with a contemporary strain of IAV (ma2009). Following intranasal inoculation with ma2009, males (n= 13-15/treatment) were monitored for changes in body mass (D), clinical disease (E), and pulmonary virus titers (F). Significant differences between testosterone-treated and placebo-treated gdx male mice are represented by asterisks and significant differences between intact and placebo-treated gdx males are represented by a dagger ($p < 0.05$).

Figure 2.5: Effects of testosterone-treatment on pulmonary inflammation following ma2009 virus infection in young adult males. Lungs were collected from young adult males that were gonadectomized and treated with either placebo (Gdx) or testosterone (Gdx + T) and either mock infected or infected with influenza A virus (ma2009) and euthanized at 5, 9, 14, or 21 days post-inoculation (dpi) (n=6-10/treatment/time-point). Lung tissues were sectioned, stained with

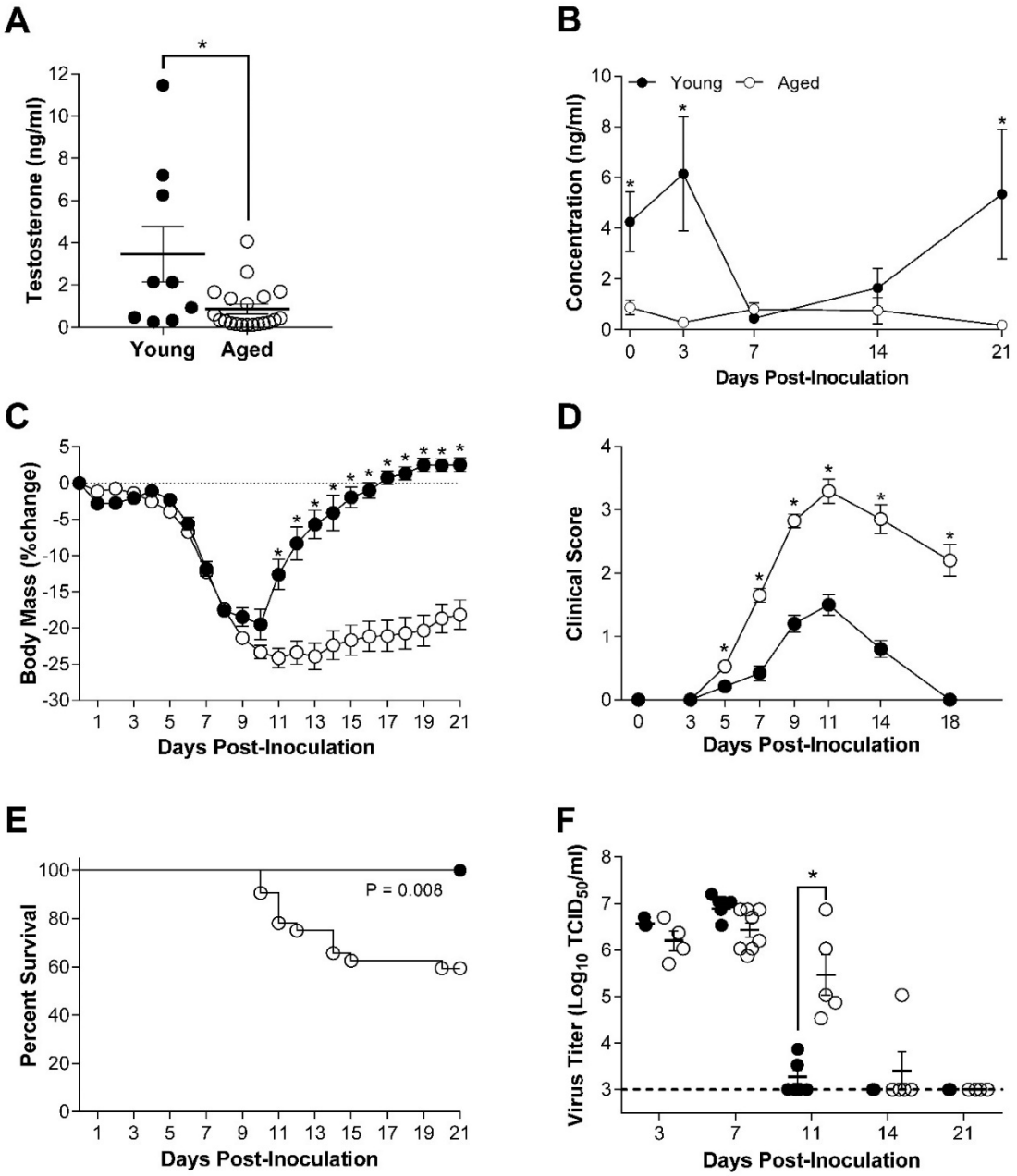
H&E, and scored for makers of inflammation, including perivascular inflammation, peribronchiolar inflammation alveolar inflammation, and edema. Cumulative inflammation scores at 5, 9, 14, or 21 dpi are presented (A). Histology scores (B) and representative photomicrographs (C) are shown for 14 dpi. Significant differences between gonadectomized young adult male mice treated with placebo or testosterone are represented by asterisk ($p < 0.05$).

Figure 2.6: Effects of testosterone-replacement on the outcome of ma2009 virus infection in aged male mice. Aged male mice were either treated with placebo (P) or time-release testosterone capsules (T) for one week prior to inoculation with influenza A virus (IAV; ma2009). Concentrations of testosterone (A) were measured in serum samples collected 21 days post-inoculation (dpi) (n=9-10/treatment). Following intranasal inoculation with IAV, body mass (B), clinical disease (C), and survival (D) were monitored for 21 dpi (n=24-34). Titers of infectious virus were measured 3, 7, 14, or 21 dpi (E; n=4-8/treatment/time-point). Lung tissue was collected from mock-infected and IAV-infected aged mice that were treated with placebo or testosterone and euthanized 7, 14, or 21 days post-inoculation (dpi) (n=5-10/treatment/time-point). Lung tissue sections were scored for makers of inflammation and cumulative inflammation scores are presented (F). Significant differences between testosterone-treated and placebo-treated aged male mice are represented by asterisks ($p < 0.05$).

Figure 2.7: Effects of age and testosterone on neutralizing and total IgG antibody responses in young and aged males infected with either PR8 or ma2009 virus. Neutralizing and total anti-influenza IgG antibody titers were measured in serum samples collected 21 days post-inoculation (dpi) with either PR8 or ma2009 in young and aged adult males (n = 4-12/virus/age;

A-D), gonadectomized young adult males treated with either placebo (Gdx) or testosterone (Gdx + T; n = 7-12/virus/treatment; E-H), or in aged male treated with either placebo (P) or testosterone (T; n = 4-10/virus/treatment; I-L). Significant differences are represented by asterisks ($p < 0.05$).

Figure 2.1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Young
Aged
B
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Concentration (ng/ml)
Days Post-Inoculation
C
Body Mass (%change)
Days Post-Inoculation
D
Clinical Score
Days Post-Inoculation
E
Percent Survival
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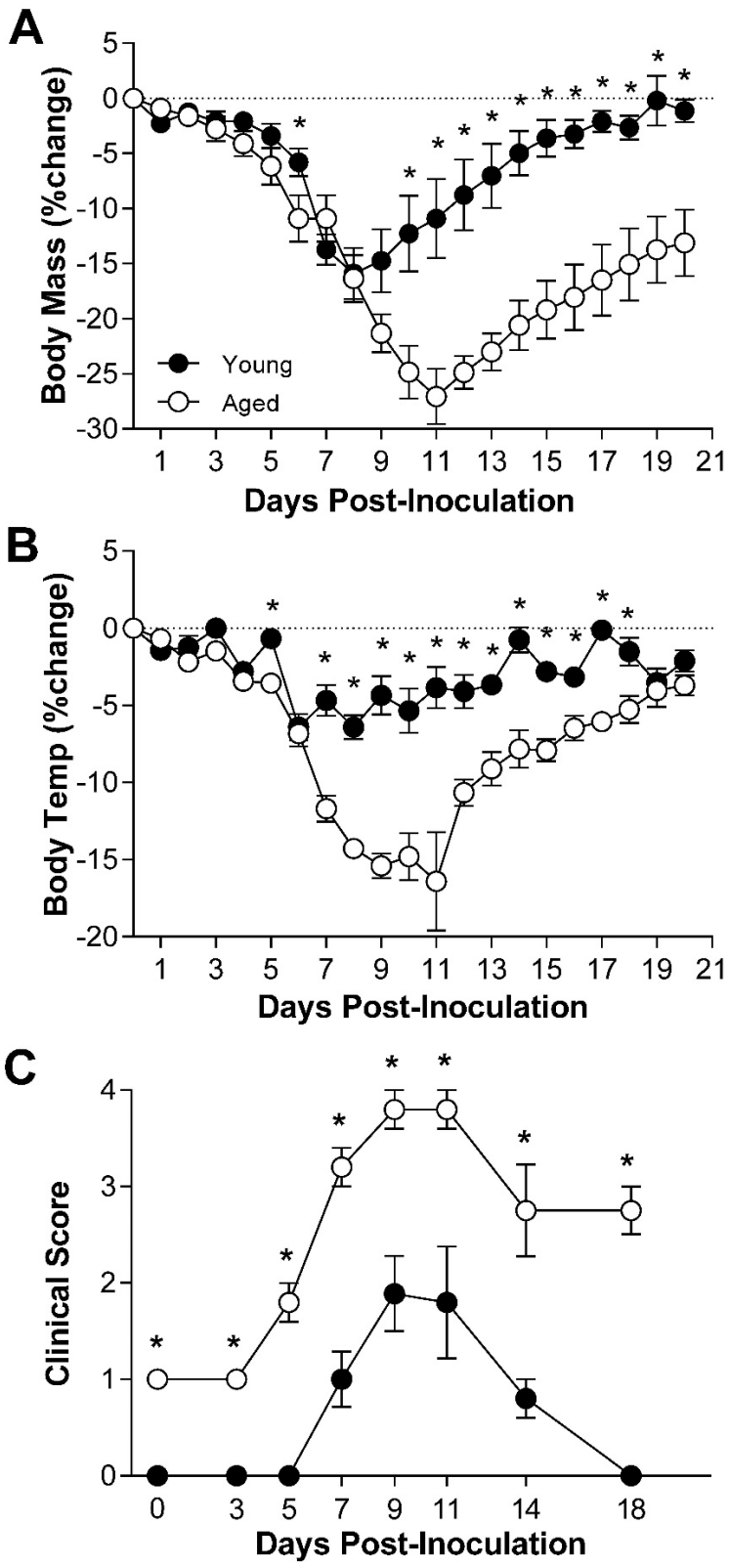
Days Post-Inoculation
F
Virus Titer (Log10 TCID50/ml)
Days Post-Inoculation

Figure 2.2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Body Mass (%change)
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Figure 2.3

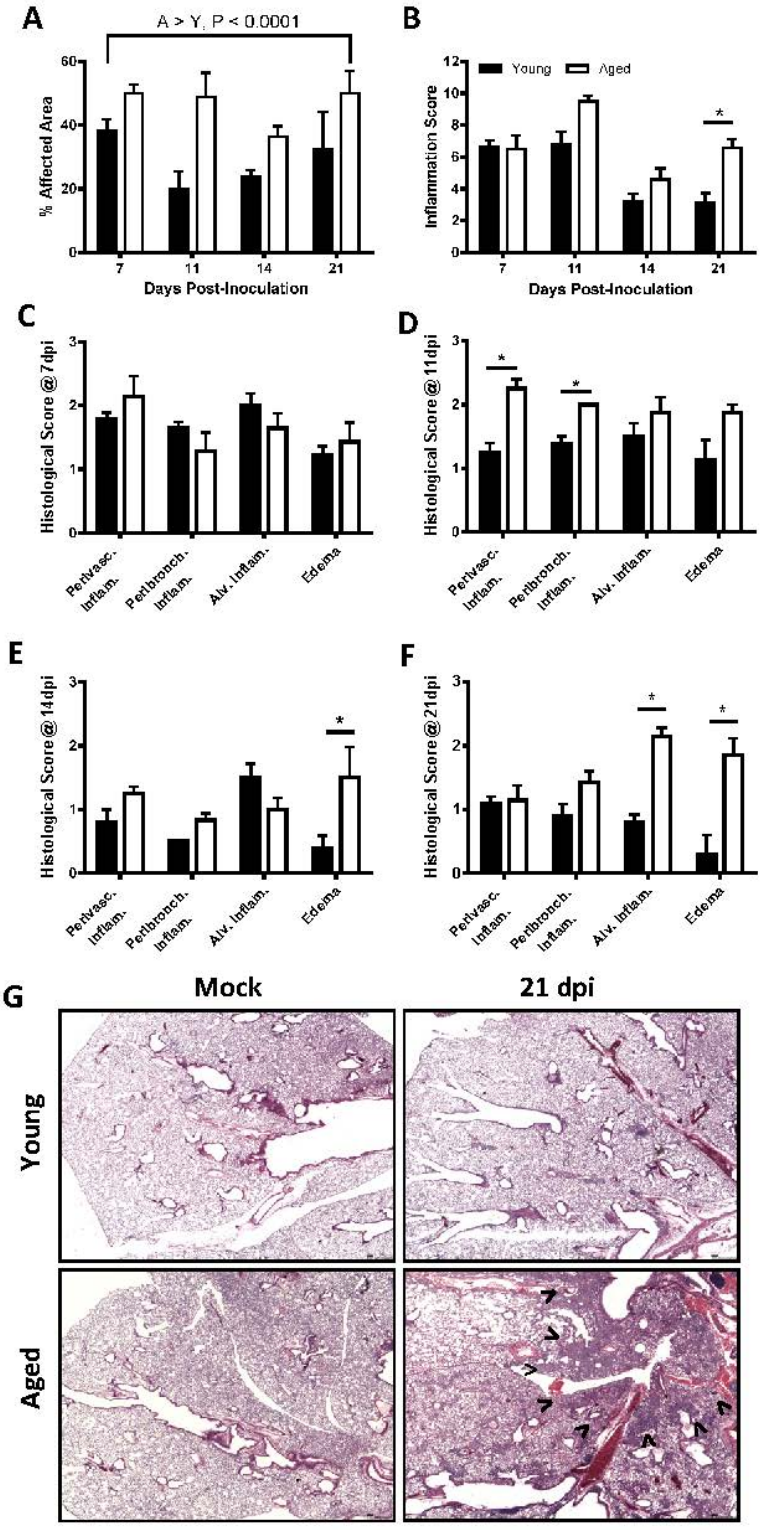

A
A > Y, P < 0.0001
% Affected Area
Days Post-Inoculation
B
Young
Aged
Inflammation Score
Days Post-Inoculation
C
Histological Score @ 7dpi
D
Histological Score @ 11dpi
E
Histological Score @ 14dpi
F
Histological Score @ 21dpi
Perivasc. Inflam.
Peribronch. Inflam.
Alv. Inflam.
Edema
G
Mock
21 dpi
Young
Aged

Figure 2.4

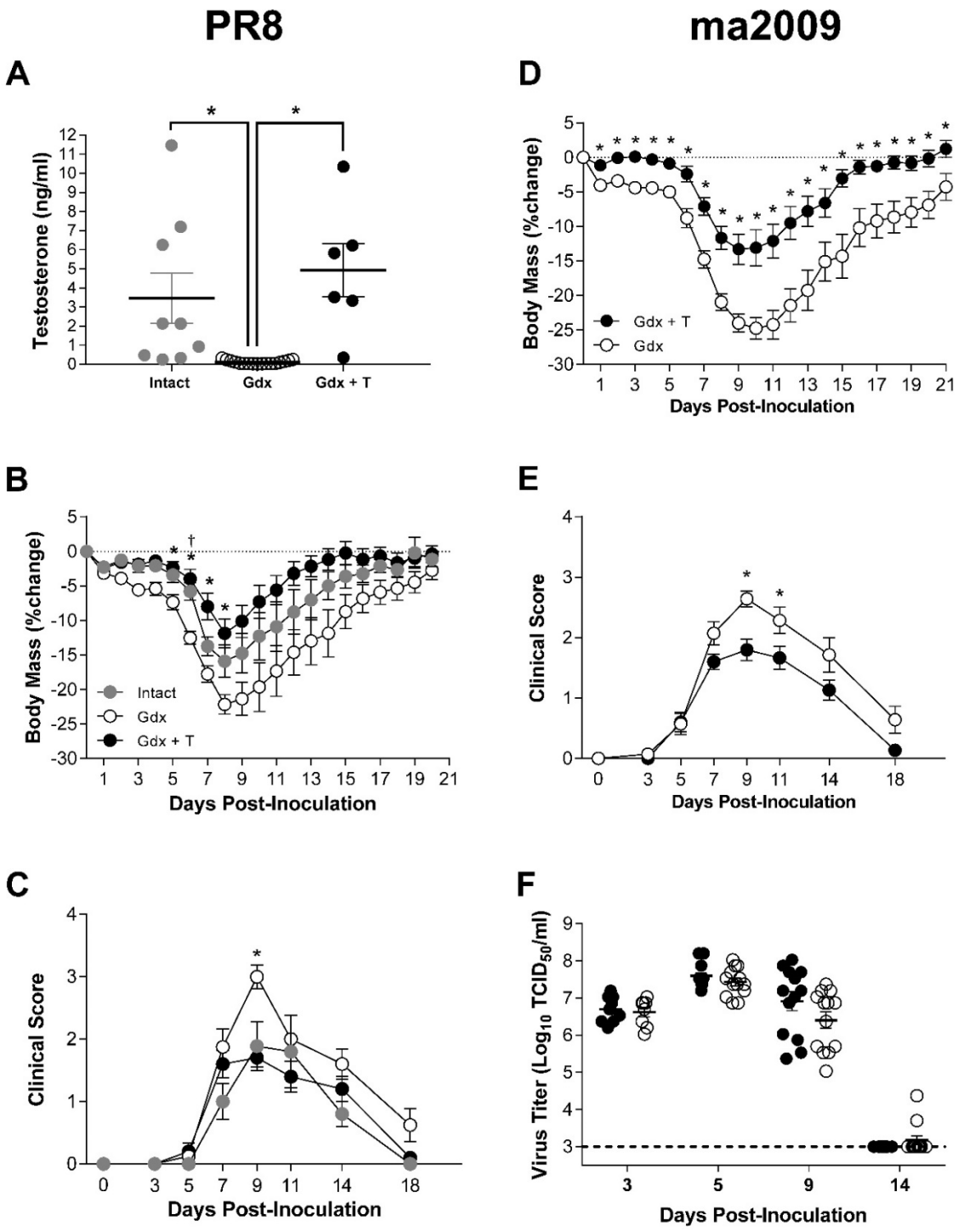

PR8
ma2009
A
Testosterone (ng/ml)
Intact
Gdx
Gdx + T
D
Body Mass (%change)
Gdx + T
Gdx
Days Post-Inoculation
B
Body Mass (%change)
Intact
Gdx
Gdx + T
Days Post-Inoculation
E
Clinical Score
Days Post-Inoculation
C
Clinical Score
Days Post-Inoculation
F
Virus Titer (Log10 TCID50/ml)
Days Post-Inoculation

Figure 2.5

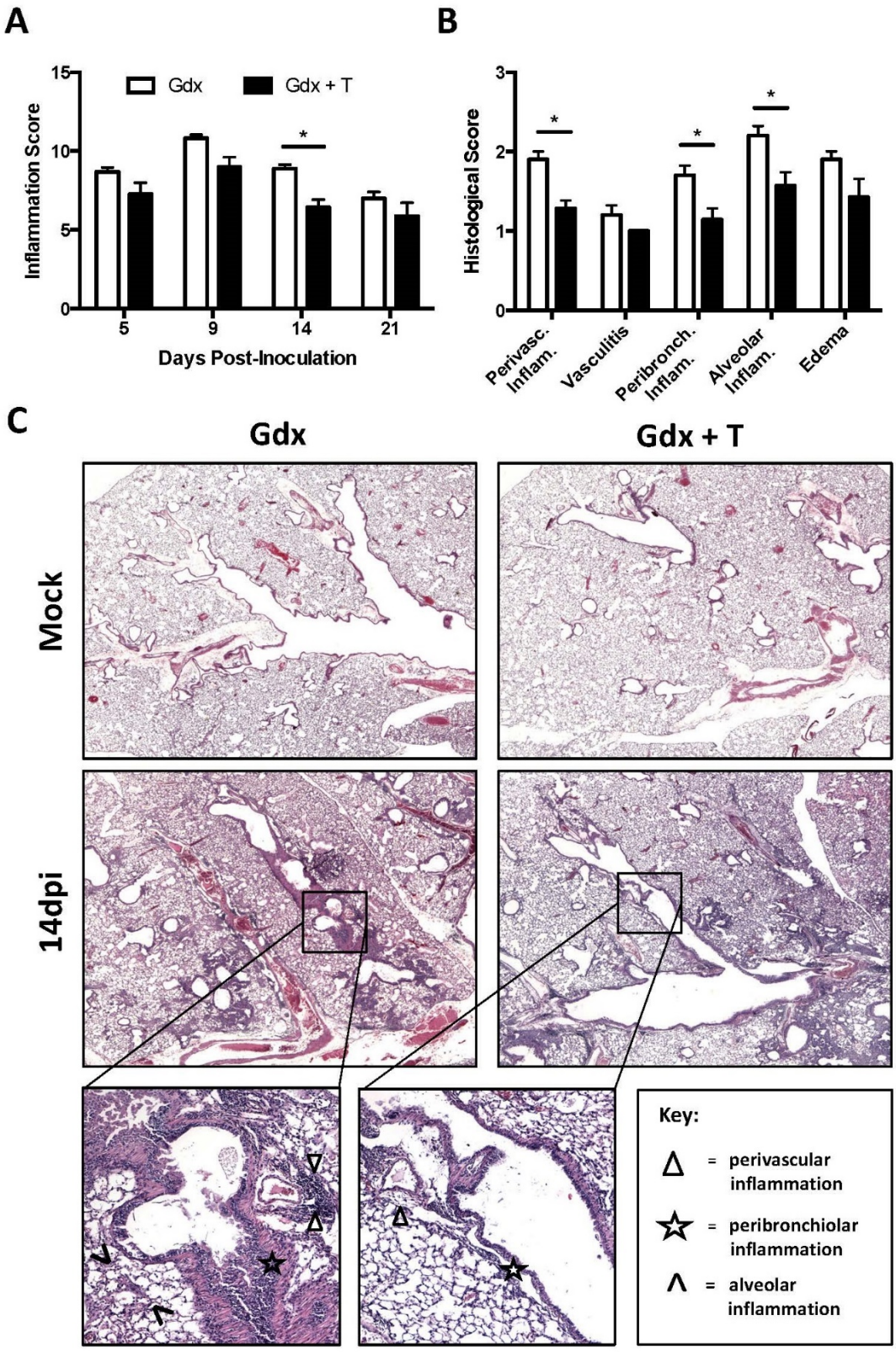

A
B
C
Gdx
Gdx + T
Inflammation Score
Days Post-Inoculation
Histological Score
Perivasc. Inflam.
Vasculitis
Peribronch. Inflam.
Alveolar Inflam.
Edema
Gdx
Gdx + T
Mock
14dpi
Key:
= perivascular inflammation
= peribronchiolar inflammation
= alveolar inflammation

Figure 2.6

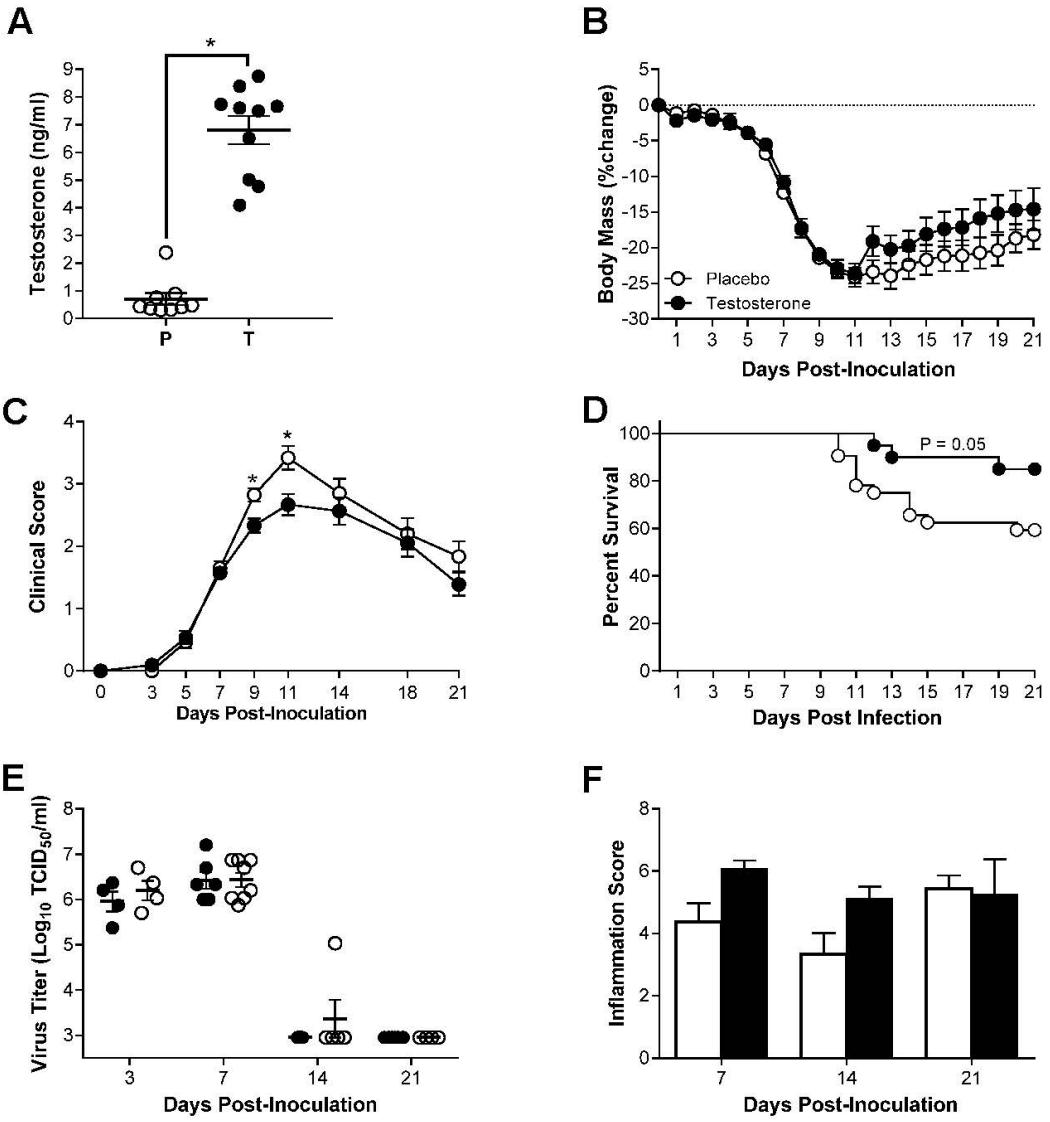
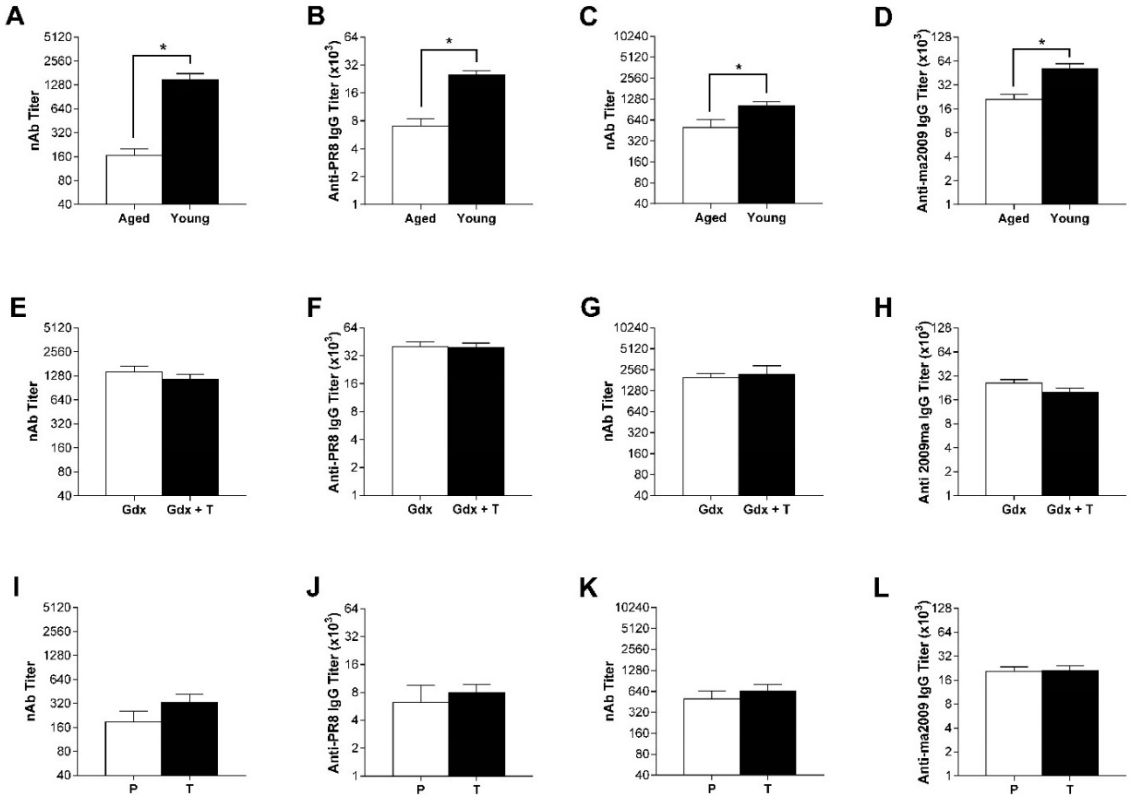

A
Testosterone (ng/ml)
P
T
B
Body Mass (%change)
Placebo
Testosterone
Days Post-Inoculation
C
Clinical Score
Days Post-Inoculation
D
Percent Survival
P = 0.05
Days Post Infection
E
Virus Titer (Log10 TCID50/ml)
Days Post-Inoculation
F
Inflammation Score
Days Post-Inoculation

Figure 2.7


A
nAb Titer
5120
2560
1280
640
320
160
80
40
*
Aged
Young
B
Anti-PR8 IgG Titer (x10³)
64
32
16
8
4
2
1
*
Aged
Young
C
nAb Titer
10240
5120
2560
1280
640
320
160
80
40
*
Aged
Young
D
Anti-ma2009 IgG Titer (x10³)
128
64
32
16
8
4
2
1
*
Aged
Young
E
nAb Titer
5120
2560
1280
640
320
160
80
40
Gdx
Gdx + T
F
Anti-PR8 IgG Titer (x10³)
64
32
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8
4
2
1
Gdx
Gdx + T
G
nAb Titer
10240
5120
2560
1280
640
320
160
80
40
Gdx
Gdx + T
H
Anti 2009ma IgG Titer (x10³)
128
64
32
16
8
4
2
1
Gdx
Gdx + T
I
nAb Titer
5120
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320
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Anti-PR8 IgG Titer (x10³)
64
32
16
8
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2
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P
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K
nAb Titer
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5120
2560
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640
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160
80
40
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Anti-ma2009 IgG Titer (x10³)
128
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Chapter 3

Androgen receptor signaling in the lungs mitigates inflammation and promotes recovery from influenza in male mice

Landon G. vom Steeg, Katherine Mulka, Emma Reilly, David J. Topham and Sabra L. Klein

In preparation for submission to *PLoS Pathogens*

Abstract

Circulating androgens in males can modulate immune cell activity, but the impact of androgens on viral pathogenesis remains unclear. Previous data demonstrate that testosterone reduces the severity of influenza A virus (IAV) infection in male mice by mitigating pulmonary inflammation rather than by affecting viral replication. To examine the immune responses mediated by testosterone to mitigate IAV-induced inflammation, adult male mice remained gonadally intact or were gonadectomized and treated with either placebo or androgen-filled (i.e., testosterone or dihydrotestosterone) capsules prior to sublethal IAV infection. Similar to intact males, treatment of gonadectomized males with androgens improved the outcome of IAV infection, which was not mediated by changes in the control of virus replication or pulmonary cytokine activity. Instead, androgens accelerated pulmonary leukocyte contraction during the recovery phase of infection (i.e., after virus clearance) to limit inflammation. To identify which immune cells were contracting in response to androgens, the composition of pulmonary cellular infiltrates was analyzed and revealed that androgens specifically accelerated the contraction of total $CD8^+$ T cells, IAV-specific $CD8^+$ T numbers, and cytokine production and degranulation by IAV-specific $CD8^+$ T cells, while also inhibiting the influx of eosinophils into the lungs following clearance of IAV. CD8+ T cells expressed androgen receptor (*Ar*) mRNA and the effects of testosterone on the contraction of $CD8^+$ T numbers and activity were blocked by co-administration of the androgen receptor antagonist, flutamide. Despite the presence of *Ar* mRNA in CD8+ T cells, adoptive transfer of CD8+ T cells revealed that the presence of testosterone in recipient rather than donor mice protected males against IAV by limiting inflammation associated with prolonged CD8+ T cell activity in the lungs. These data suggest that testosterone creates an environment that promotes downregulation of detrimental inflammatory immune responses to protect against IAV.

Introduction

Testosterone, a sex steroid hormone produced and released primarily by Leydig cells in the testes of males, has significant effects on health and disease [430]. In men, low testosterone, whether congenital, acquired, or age-related, is associated with an increased risk of all-cause and cardiovascular related mortality [35, 40, 41]. Additionally, low testosterone in males has been linked to metabolic dysfunction, osteoporosis, muscle weakness, fatigue, cognitive impairment, and sexual dysfunction; while in hypogonadal men, testosterone replacement therapy has been shown to improve cardiovascular disease outcomes, increase quality of life perceptions, and improve age-associated anemia [41, 44-48]. Although safety concerns exist (e.g., cardiovascular disease risk), the perceived benefits of testosterone replacement therapies have resulted in a dramatic increase in its therapeutic use over the last two decades, with an estimated 2.3 million men undergoing testosterone replacement therapy in the United States alone in 2013 [49, 50]. Included in these numbers is a 4-fold increase in testosterone replacement therapy use in reproductively aged males (i.e. 18 to 45 years of age), a demographic often overlooked in studies of the implications of low testosterone [53]. Despite the increasing popularity of testosterone replacement therapy, the influence of testosterone deficiency and treatment on clinical outcomes of infectious disease has not been adequately considered.

The biological effects of testosterone are typically mediated through androgen receptor (AR) signaling [5, 35]. Intracellular androgen receptors are present in cells throughout the body, with testosterone modulating the activities of a variety of tissue and cell types [35]. Notably, ARs are widely expressed in cells of both the innate and adaptive immune system, including macrophages, neutrophils, and T cells [5, 35]. In humans and nonhuman animals, testosterone and its physiologically active metabolite, dihydrotestosterone (DHT), are broadly

immunoregulatory and capable of altering the number, function, and differentiation of most immune cell populations. For example, in the presence of testosterone, murine macrophages increase IL-10 and decrease TNFα synthesis, while T cell numbers and activity (e.g., IL-4 and Il-12 production) are reduced [70, 121]. In adult human males, clinical depletion of testosterone decreases regulatory T cell numbers (Tregs), reduces mitogen-induced IFNγ expression in $CD8^+$ T cells, and suppresses the ability of natural killer cells to proliferate [91]. Although, the immunomodulatory properties of testosterone are well known, the impact of low testosterone on the severity of viral infection remains incompletely characterized. If testosterone is capable of broadly regulating the immune system, then in viral infections where pathogenesis is driven by the immune response rather than viral replication, testosterone is likely to protect and reduce the severity of infection.

Disease following IAV infection is largely immune-mediated, with severe disease often associated with excessive or aberrant immune responses (i.e., a cytokine storm) to the virus [228, 267]. We have previously shown that low testosterone in males, whether age-related or surgically-induced, increases the severity of IAV infection [164, 431]. Furthermore, these changes were associated with delayed resolution of pulmonary inflammation, independent of either changes in viral replication or induction of growth factors (e.g., amphiregulin), suggesting that the protective effects of testosterone are mediated through changes in the inflammatory response to infection [302, 432]. In the current study, we sought to characterize the effects of testosterone on the immune response to IAV using a murine model of IAV infection. We show that testosterone improves the outcomes of IAV infection not by mitigating the cytokine storm, but by promoting the contraction of virus-specific pulmonary $CD8^+$ T cells following control of viral replication. The effects of testosterone were dependent on androgen receptor signaling.

Despite CD8+ T cells expressing the androgen receptor, the protective effects of testosterone on IAV pathogenesis were mediated by indirect, rather than direct, effects on CD8+ T cells.

Materials and Methods

Animals

All animal procedures were approved by the Johns Hopkins Animal Care and Use Committee (MO18H262). Adult (7-8 weeks old) male C57BL/6 mice were purchased from Charles River. For adoptive transfer experiments, male and female TCR-Ova (C57BL/6-Tg(TcraTcrb) 1100MjbJ/J) and CD90.1 (B6.PL-*Thy1* ^{a}/CyJ) mice were purchased from The Jackson Laboratory as breeding pairs and bred in house to obtain male offspring. All mice were housed at 3-5 animals per microisolator cage under standard BSL-2 housing conditions and given food and water *ad libitum*.

Gonadectomy and hormone manipulation

Adult (8 week old) male mice were anesthetized by intra-peritoneal inoculation with a ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail and the testes were removed bilaterally as described previously [164]. Following two weeks recovery, silastic tubing capsules (inner diameter-0.04", outer diameter- 0.085"; HelixMark) containing crystalline testosterone propionate (7.5 mm; Sigma), crystalline 4,5α-Dihydrotestosterone (5.0 mm; Sigma), or nothing were implanted subcutaneously [433]. For flutamide studies, capsules were prepared as above (2 x 15.0 mm; Sigma) but were implanted at the time of gonadectomy. All capsules were sealed with 2.5 mm of medical adhesive (Factor II, A-100) and incubated at 37°C overnight in sterile saline solution prior to implantation.

Virus infection and quantification

Mouse-adapted A/California/O4/09 (ma2009; H1N1; generated by Dr. Andrew Pekosz using a published sequence) [401] or recombinant A/WSN/33 virus containing $OVA_{257-264}$ (SIINFEKL)

peptide in the neuraminidase protein (H1N1; WSN-Ova_1) [434], were used in all experiments. Mice were anesthetized and infected by intranasal inoculation with ma2009 or WSN-Ova_1 H1N1 virus (ma2009 = 0.1 MLD_{50}; WSN-OVA_1 = 0.4 MLD_{50}) diluted in 30µl of DMEM or mock infected with 30µl DMEM. For virus quantification, log_{10} dilutions of lung homogenate were plated onto Madin-Darby canine kidney (MDCK) cell monolayers in replicates of 6 for 5 days at 32°C. Cells were stained with naphthol blue black (Sigma Aldrich) and scored for cytopathic effect. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the Reed-Muench method and was used to back titer all viral inoculums.

Sample collection and testosterone quantification

Following infection, rectal temperature and body mass were recorded daily out to 21 days post inoculation (dpi), and clinical disease scores were recorded at select time-points as described previously [432]. For terminal studies, mice were euthanized at select time-points and plasma, whole lungs, spleen, and mediastinal lymph nodes (MLN) were collected. Seminal vesicles were also collected, and mass was recorded as a bio-marker for androgen activity. Total testosterone concentration was quantified in plasma collected at 21 dpi by commercial ELISA kit according to the manufacturer's instructions (IBL America). To prevent sample degradation, care was taken to limit light and thermal exposure of plasma samples prior to testosterone quantification.

Pulmonary cytokine and chemokine quantification

Snap-frozen lung tissue was homogenized in DMEM supplemented with 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco) and centrifuged to remove cellular debris. Supernatants were collected and Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, CXCL-1, MCP-1, MIP-1α, MIP-1β, RANTES, and TNFα were

quantified using the Bio-Plex Pro Mouse Cytokine 23-Plex Assay (Biorad) according to the manufacturer's instructions. Pulmonary TGFβ concentration was quantified by commercial ELISA kit (R&D Systems). For analyses, IL-9 and IL-17A concentrations remained below the limit of detection at all time-points and were excluded.

Flow cytometry

Lung, spleen, and MLN tissues were harvested, and single cells suspensions were generated by homogenizing tissue through a 100µm nylon filter (Falcon) followed by ACK lysis of red blood cells (Quality biologicals). The total numbers of live cells were determined using a hemocytometer and trypan blue (Invitrogen) exclusion, and cells were resuspended at 1×10^6 cells/ml in RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (Fisher Scientific), 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). For the enumeration of H1N1-specific $CD4^+$ and $CD8^+$ T cells, isolated cells were cultured for 5hrs at 37°C in media containing IAV specific peptide (2009ma; CD8: $NP_{366-374}$, or CD4: $NP_{311-325}$, and WSN-OVA_1; CD8: $OVA_{257-264}$) in the presence of GolgiPlug (BD) and GolgiStop (BD). Following incubation, T cell viability was determined by fixable live/dead far red viability stain (Invitrogen). For all leukocyte populations, Fc receptors were blocked using anti-CD16/32 (BD Biosciences) and panel specific surface markers were stained with the following antibodies: CD4-PerCPCy5.5 (Clone RM1-5; BD), CD8-PerCPCy5.5 (Clone 53-6.7; BD), CD11b-FITC (Clone M1/70; BD), CD11C-APC (Clone HL3; BD), CD25-FITC (Clone 7D4; BD) CD45-PerCPCy5.5 (Clone 30-F11; BD), CD90.1-FITC (Clone OX-7; BD), CD90.2-PE (Clone 53-2.1; BD), CD107a-PE (Clone 1D4B; BD), Ly-6G-FITC (Clone 1A8; BD), I-A/I-E (Clone M5/114.15.2), Siglec-F (Clone E50-2440), PE-conjugated tetramer for ma2009 (ASNENVETM; NIH Tetramer Core Facility), and PE-conjugated pentamer for WSN-OVA_1 (SIINFEKL; Proimmune). Cells were then permeabilized and fixed (BD Cytofix/Cytoperm) prior to intracellular staining with IFNγ-FITC (Clone XMG1.2; BD), IL4-PE (BD), IL17A-PE (BD), and TNFα-

PE (Clone MP6-XT22; BD). Intracellular staining with Foxp3 (Clone MF23; BD) was performed following fixation and nuclear permeabilization with the FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Data were acquired using a FACSCailubr flow cytometer (BD) running Cell Quest Pro and analyzed using FlowJo (v.10) software (Tree Star, Inc.). Total cell counts were determined based on the total live cells counts acquired by trypan blue exclusion staining multiplied by the total live cell percentages for each corresponding gate.

Real time reverse transcription PCR

Pulmonary single cells suspensions were generated by homogenizing lung tissue through a 100µm nylon filter (Falcon) and $CD8^+$ T cells were isolated by negative selection (StemCell Technologies). Total RNA was then isolated from purified $CD8^+$ T cells using a commercial kit (Invitrogen) per the manufacturer's instructions and RNA concentration and purity were measured using a NanoDrop (ThermoFisher Scientific). Pre-designed androgen receptor (*Ar)* (Mm.PT.58.12425400) and *Gapdh* (Mm.PT.39a.1) PrimeTime Primers were purchased from Integrated DNA Technologies. Semi-quantitative RT-PCR was performed in 96-well optical reaction plates using SsoFast EvaGreen Supermix (Biorad) on the StepOnePlus RT-PCR system (Applied Biosystems). Gene expression was normalized to *Gapdh* and mock-infected samples using the ΔΔCt method.

Adoptive Transfer of $CD8^+$ T cells

Splenic tissue was harvested from unprimed gonadectomized male TCR-Ova mice treated with either empty capsules (gdx) or testosterone capsules (gdx + T). $CD8^+$ T cells were isolated by tissue homogenization through a 100µm nylon filter (Falcon), followed by negative selection purification (StemCell Technologies). One-hundred thousand purified $CD8^+$ T cells were then

transferred into gdx and gdx + T treated naive male CD90.1 recipient mice via intravenous inoculation. Recipient mice were infected with WSN-Ova_1 virus 48 hours post transfer.

Statistical Analysis

Discrete measures were analyzed by one or two-way ANOVA with significant interactions further analyzed using the Tukey method for pairwise multiple comparisons. Repeated measures were analyzed by mixed-effect model with Bonferroni's post-test for multiple comparisons. Statistical analyses were performed using GraphPad Prism 8.00 software and mean differences were considered significant at $P < 0.05$.

Results

Testosterone reduces the severity of IAV infection in male mice

To assess the effects of testosterone on the severity of influenza virus infection, adult male mice underwent sham surgeries or were gonadectomized and received either testosterone or placebo capsules. Gonadectomized males had significantly lower concentrations of circulating testosterone and seminal vesicle mass than either gonad-intact or gonadectomized males that received testosterone (**Fig 3.1A** and **3.1B**; $p < 0.05$). Following intranasal inoculation with a sub-lethal dose of ma2009 H1N1, mice were monitored for 21 days post inoculation (dpi) for changes in body mass, body temperature, and clinical disease severity. Similar to previous studies [164, 432], testosterone-depleted mice experienced greater body mass loss, hypothermia, and clinical disease severity than either gonad-intact or gonadectomized males that received testosterone (**Fig 3.1C-1E**; $p < 0.05$). Despite these differences in the severity of IAV infection between testosterone-depleted and testosterone-replete mice, neither peak virus titers at 7 dpi nor the clearance of infectious virus from the lungs by 14 dpi was affected by testosterone concentrations in males (**Fig 3.1F**). These data illustrate that testosterone reduces the severity of IAV infection, independent of changes in viral replication.

Pulmonary cytokine and chemokine concentrations are not altered by testosterone in males

The severity of IAV infection is associated with profound induction of pulmonary cytokine and chemokine responses, which can lead to excessive cellular infiltration, pulmonary inflammation, and tissue damage, if improperly regulated [269, 270]. To test whether testosterone altered the kinetics or magnitude of the cytokine and chemokine response during IAV infection, pulmonary concentrations of 24 cytokines and chemokines were measured at selected time-points. Pulmonary concentrations of pro-inflammatory cytokines and chemokines (e.g. IL-6, G-CSF, and

CCL2) broadly increased in response to infection at 3 and 9 dpi, and then declined following control of viral replication at 14 dpi (**Fig 3.2A-2E** and **SI Table 3.1**). The only chemokine that was significantly altered by testosterone treatment was CXCL1, which was greater in the lungs of testosterone-treated, than testosterone-depleted male mice at 3 and 14, but not 9 dpi (**SI Table 3.1**; $p < 0.05$). Despite the known anti-inflammatory effects of testosterone [125, 435, 436], testosterone treatment did not alter pulmonary concentrations of either IL-10 or TGFβ during IAV infection (**Fig 3.2F** and **SI Table 3.1**). Taken together, these data suggest that the improved outcome of IAV associated with testosterone is independent of substantial changes in the 'cytokine storm' during IAV infection.

Testosterone alters the influx and contraction of pulmonary immune cells during the resolution of IAV infection

Differences in the numbers and kinetics of immune cells that influx into the lungs during infection can greatly impact IAV pathogenesis [272-274]. To test the hypothesis that testosterone affected immune cell recruitment into the lungs during IAV infection, total numbers of innate and adaptive immune cells were enumerated in the lungs of testosterone or placebo-treated gonadectomized male mice. The total number of leukocytes (i.e., $CD45^+$ cells) in the lungs peaked at 7 dpi (i.e., during peak virus replication) and was followed by a greater decline in cell numbers in the lungs of testosterone-treated relative to placebo-treated males at 14 and 21 dpi (i.e., after control of virus replication) (**Fig 3.3A**; $p < 0.05$).

To identify which immune cell types persisted in the lungs of testosterone depleted males, we further characterized the composition of pulmonary cellular infiltrates. During IAV infection, the numbers of interstitial macrophages, neutrophils, plasmacytoid dendritic cells, and conventional dendritic cells increased in both testosterone- and placebo-treated

gonadectomized males, with peak numbers of innate immune cells in the lungs occurring at 7 dpi (**Table 3.1**). In contrast, the number of alveolar macrophages declined over the course of infection in all male mice (**Table 3.1**).

The only cell types affected by testosterone treatment in males were neutrophils and eosinophils. The number of pulmonary neutrophils was transiently greater at baseline (0 dpi) and 3pi, but not at other dpi, in gonadectomized males that were treated with testosterone as compared with those treated with placebo (**Table 3.1**; $p < 0.05$). In contrast, a significant influx of eosinophils into lungs occurred in gonadectomized males treated with placebo, but not testosterone, after clearance of virus from the lungs (i.e., 14 and 21 dpi) (**Fig 3.3B**; $p < 0.05$). The influx of eosinophils into the lungs of testosterone depleted males during the resolution phase of infection may be indicative of eosinophilic pneumonia [437], which may contribute to the pulmonary histological inflammation reported previously in testosterone depleted male mice [432].

In addition to affecting innate immune cells, testosterone is associated with shifts in the numbers, activities, and differentiation of $CD4^+$ T cells in experimental models of allergy and autoimmune disease [117, 122, 125, 438]. To test the hypothesis that testosterone improved the outcome of IAV infection by shifting populations of $CD4^+$ T cells, helper T cell type 1 (Th1), type 2 (Th2), type 17 (Th17), and regulatory T (Treg) cells where quantified at several time points before and during IAV infection. Peak numbers of total $CD4^+$ T cells, Th1, Th2, Th17, and Treg cells occurred in both testosterone-depleted and -replete males at 9 dpi, followed by a retraction of cell numbers at 14 dpi (**Table 3.2**). The contraction of total $CD4^+$ T cell numbers in the lungs was significantly slower of testosterone-depleted as compared with replete males at 14 and 21 dpi (**Table 3.2**; $p < 0.05$). There was no effect of testosterone treatment on the numbers of IAV-specific Th1, Th2, or Th17 cells at any time point examined (**Table 3.2**). In

contrast with previous reports of testosterone-induced expansion of Treg cell numbers [122], Treg cell numbers were greater in the lungs of placebo-treated relative to testosterone-treated gonadectomized males, but only at 21 dpi (**Table 3.2**; $p < 0.05$). These data suggest that $CD4^+$ T cells are not the primary cell type mediating the protective effects of testosterone during IAV infection of male mice.

Virus-specific $CD8^+$ T cells are beneficial for the killing of IAV-infected cells but can also be detrimental to the host by causing immunopathology [275, 277]. Total $CD8^+$ as well as IAV-specific $CD8^+$ T cells influxed into the lungs at 9 dpi (**Fig 3.3C** and **3D**), which corresponded with peak virus titers (**Fig 3.1F**). Testosterone had no effect on either the induction or peak magnitude of total or virus-specific $CD8^+$ T cells in the lungs; the contraction of these cells, however, following virus clearance (14 and 21 dpi) was significantly better in testosterone-treated as compared with placebo-treated gonadectomized males (**Fig 3.3C** and **3.3D**; $p < 0.05$ in each case). To determine if testosterone suppressed the activity of virus-specific $CD8^+$ T cells, we assessed cytokine protection following *ex vivo* stimulation with ma2009 H1N1-specific peptide and observed that the number of $CD8^+$ T cells producing IFNγ was significantly reduced in testosterone treated males relative to testosterone depleted males after virus had been cleared (i.e., 14 and 21 dpi) but not during peak virus titers (i.e., 9 dpi; **Fig 3.3E**; $p < 0.05$). To further assess whether testosterone affected the functionality of IAV-specific $CD8^+$ T cell we used CD107a as a marker for degranulation in response to *ex vivo* ma2009 H1N1-specific peptide stimulation. After virus had been cleared from the lungs, the contraction in the numbers of $CD8^+$ T cells staining positive for both surface expression of CD107a and production of IFNγ was delayed in the lungs of testosterone-depleted relative to testosterone-replete male mice (**Fig 3.3F**; $p < 0.05$). The effects of testosterone on the contraction of IAV-specific $CD8^+$ T cells after virus had been cleared from the lungs, was specific to the site of virus replication and not

observed in either the spleen or the mediastinal lymph nodes (i.e. pulmonary draining lymph nodes; **Table 3.3**). Taken together, these data suggest that testosterone may improve the outcome of IAV infection by dampening immunopathology caused by pulmonary virus-specific $CD8^+$ T cell populations.

The protective effects of testosterone during IAV infection are dependent on androgen receptor signaling

Testosterone can be metabolized in tissues, converted into estradiol, and signal through estrogen receptors [439]. Estradiol signaling through estrogen receptor α can dampen inflammation and improve the outcome of IAV infection, at least in female mice [164, 418, 440]. To determine whether the protective effects of testosterone during IAV infection in male mice were caused by signaling through androgen or estrogen receptors, male mice were gonadectomized and implanted with capsules containing either testosterone, placebo, or a combination of testosterone and the androgen receptor antagonist flutamide [441]. Seminal vesicle mass was used as a biomarker to confirm androgen receptor inhibition by flutamide and was significantly reduced in males that received testosterone + flutamide treatment as compared with males that received testosterone alone (**Fig 3.4A**; $p < 0.05$). During IAV infection, flutamide treatment inhibited the protective effects of testosterone on morbidity (**Fig 3.4B** and **3.4C**; $p < 0.05$). To assess whether the testosterone-induced changes in $CD8^+$ T cell numbers and activity were also androgen receptor-dependent, we evaluated the effects of flutamide on the contraction of $CD8^+$ T cells during the resolution phase of infection. Co-treatment of flutamide and testosterone, similar to placebo treatment, resulted in significantly greater numbers of total $CD8^+$ T cells, ma2009 H1N1-specific $CD8^+$ T cell, and virus-specific $CD8^+$ T cells producing IFNγ in

response to H1N1 specific peptide stimulation at 14 and 21 dpi as compared with testosterone treatment alone (**Fig 3.4D-4F**; $p < 0.05$).

Because flutamide can alter T cell function through off-target GABA-A receptor signaling [442], we sought to confirm the effects of androgen receptor signaling on IAV pathogenesis by using the non-aromatizable androgen, dihydrotestosterone (DHT) (i.e., an androgen that cannot be converted into estradiol). Treatment of gonadectomized males with DHT significantly increased seminal vesicle mass relative to placebo-treated males, to a mass consistent with testosterone-treated males (**Fig 3.5A**; $p < 0.05$). Males that were gonadectomized and treated with DHT prior to IAV infection were protected against IAV and experienced a similar level of morbidity (i.e., body mass and temperature loss) as testosterone-treated males, which was collectively better than placebo-treated mice (**Fig 3.5B** and **3.5C**; $p < 0.05$). Consistent with testosterone, DHT accelerated the contraction of total numbers of $CD8^+$ T cells, ma 2009 H1N1-specific $CD8^+$ T cell numbers, and the number of $CD8^+$ T cells producing IFNγ in response to *ex vivo* H1N1-specific peptide stimulation relative to testosterone depleted male mice at 14 and 21 dpi (**Fig 3.5D** and **3.5E**; $p < 0.05$). These data demonstrate that the protective effects of testosterone on IAV pathogenesis are dependent on androgen receptor signaling in the lungs.

Testosterone creates a local environment to promote the contraction of $CD8^+$ T cells following control of IAV replication

Testosterone can act both directly and indirectly to alter the biological activities of T cells [113, 125, 443]. For testosterone to have direct effects on the contraction of $CD8^+$ T cells during IAV infection, androgen receptor expression would need to occur within these cells. Consistent with previous reports [97, 125, 128, 129], Androgen receptor (*Ar*) mRNA was expressed in

enriched splenic $CD8^+$ T cells from both testosterone-depleted and testosterone-replete male mice, with no effect of testosterone treatment on *Ar* mRNA expression (**Fig 3.6A**).

Because *Ar* was expressed by splenic $CD8^+$ T cells, we hypothesized that testosterone could directly signal in these cells to induce contraction following control of viral replication. To test this hypothesis, TCR-Ova donor mice and CD90.1 recipient mice were gonadectomized and implanted with capsules containing either testosterone or placebo. Enriched splenic $CD8^+$ T cells from naïve TCR-Ova mice were then adoptively transferred into either placebo- or testosterone-treated CD90.1 recipient mice prior to infection with WSN-Ova_1 H1N1 IAV. Seminal vesicle mass was greater in testosterone-treated recipient mice relative to placebo-treated recipient mice for all treatment groups (**Fig 3.6B**; $p < 0.05$). Recipient males that were gonadectomized and treated with testosterone prior to IAV infection were protected against IAV and experienced similar levels of morbidity (i.e., temperature loss), regardless of the hormonal milieu of the donor transferred $CD8^+$ T cells treatment (**Fig 3.6C**; $p < 0.05$). Testosterone treatment of recipient mice accelerated the contraction of total numbers of leukocytes (i.e., $CD45^+$ cells), eosinophils, $CD8^+$ T cells, and WSN-Ova_1 H1N1-specific $CD8^+$ T cell relative to testosterone-depleted recipient male mice at 14dpi (**Fig 3.6D-6G**; $p < 0.05$), regardless of the hormonal environment of the donor transferred CD8+ T cells. Moreover, the contraction of adoptively transferred $CD90.1^+$ $CD8^+$ T cells producing IFNγ in response to *ex vivo* Ova-specific peptide stimulation was accelerated in testosterone-treated recipient mice at 14 dpi (**Fig 3.6H**; $p < 0.05$) relative to testosterone-depleted recipient mice, irrespective of the donor mouse treatment. These data demonstrate that testosterone creates a local environment that promotes accelerated contraction of virus-specific $CD8^+$ T cells.

Discussion

Inflammatory immune responses, including cytokine production and activity of virus-specific $CD8^+$ T cells, are required to control IAV infection, but if improperly regulated can contribute to tissue damage and severe outcomes [267, 268, 274, 444]. In the current and previous studies [164, 432], androgens, including testosterone and dihydrotestosterone, in male mice reduces the severity of IAV infection by promoting the resolution of pulmonary inflammation rather than by affecting viral replication. The improved resolution of IAV-induced inflammation [432] in androgen-treated males was not cause by suppression of the cytokine storm, but rather by mitigation of pulmonary $CD8^+$ T cells after virus was cleared. The effect of androgens on the resolution of $CD8^+$ T cell activity was dependent on androgen receptor signaling in the lungs, which indirectly reduced $CD8^+$ T cell numbers and activity in the lungs following clearance of IAV.

We and others [164, 302, 432] have shown that males experience less severe disease and recover faster from IAV than females. Data from the current study and others [302, 432] illustrate that testosterone limits pulmonary inflammation during IAV infection. In addition to reduced inflammation, males also repair damaged tissue faster than females which is mediated by greater production of epidermal growth factor amphiregulin in males than females [302]. Testosterone does not regulate production of amphiregulin; thus, elevated levels of both testosterone and amphiregulin contribute to improved IAV outcomes in males than females.

In the current study, the depletion of testosterone resulted in the accumulation of eosinophils in the lungs following control of viral replication, which was unexpected given the lack of observed changes in pulmonary concentrations of IL-5, IL-13, and eotaxin. Eosinophils are androgen responsive despite the absence of androgen receptor expression [24, 54, 83], with testosterone-mediated differences in eosinophilic airway responses instead being attributed to the actions Type II innate lymphoid cells (ILC2s) [88, 89]. Though not evaluated in this study,

androgens have shown to inhibit the maturation ILC2s, while decreasing IL-5 production and eosinophilic responses in murine models of airway inflammation [88, 89]. Although the precise role of eosinophils in the immune response to IAV are unclear, previous studies in mice show accumulation of eosinophils in the lungs following control of viral replication [445, 446]. Whether the accumulation of eosinophils during the resolution phase of infection represents the activation of type 2 tissue repair responses [447], or a pathological response contributing to immunopathology warrants further study.

In response to other inflammatory diseases, including experimental autoimmune encephalomyelitis, testosterone is associated with an expansion of Th2 and Th17 cell populations and suppression of Th1 activity [120, 122, 448]. During IAV pathogenesis, testosterone treatment of males did not lead to shifts in the differentiation of $CD4^+$ T helper cell population. Furthermore, although testosterone treatment can promote the expansion in numbers and activation of Treg cells in murine models of inflammation [122-124], there was only a transient effect of testosterone on Treg cells during IAV infection. Whether this represents differences in the polarizing effects of infection versus other inflammatory states should be considered.

During IAV infection, $CD8^+$ T cells play a critical role in the control of IAV infection through the production of cytokines and the removal virus-infected cells [449]. Improper regulation or prolonged activation of virus-specific $CD8^+$ T cell responses, however, can also cause immunopathology and severe pulmonary tissue damage [275, 277]. Both in humans and mice, testosterone alters the numbers, cytokine production, and proliferative potential of $CD8^+$ T cells [91, 113]. Consistent with these observations, androgens, including testosterone, accelerated the contraction of virus-specific $CD8^+$ T cells in the lungs, but not in the spleen or mediastinal lymph nodes. The significance of this tissue specific effect is unknown, but whether these effects

of testosterone on virus-specific $CD8^+$ T cells involve activation-induced cell death or inhibitory pathways warrants future study.

Testosterone can be metabolized by aromatase into estradiol to signal through estrogen receptor α, which can dampen inflammation and improve the outcome of IAV infection in females [164, 418, 439, 440]. Moreover, in male mice gonadectomized prior to the onset of puberty, castration-mediated protection against lethal IAV infection is reversed by testosterone treatment and subsequent conversion to estradiol, but not by treatment with non-aromatizable DHT [450]. In the present study, the protective effects of testosterone on IAV pathogenesis were dependent on androgen receptor signaling in the lungs. The discordant findings about the impact of androgens on IAV pathogenesis in prepubertal versus adult mice likely represent differences in developmental maturity.

The dependence on androgen receptor signaling for accelerated contraction of virus-specific $CD8^+$ T cells and the expression of *Ar* in enriched splenic $CD8^+$ T cell populations suggested that testosterone might be acting directly on these CD8+ T cell to mitigate IAV pathogenesis. Adoptive transfer studies were conducted and revealed that the presence of testosterone in the recipient mice was a better predictor of IAV outcome and contraction of virus-specific $CD8^+$ T cells than the presence of testosterone in the donor mice. These data suggest that testosterone is not acting directly on virus-specific $CD8^+$ T cells to induce intrinsic changes in these cells via androgen signaling. Instead, testosterone induces transient changes in these cells that are dependent on the presence of testosterone in the local environment in which they reside. Given the widespread expression of androgen receptors both in immune cells and epithelial cells in the lung [54, 451], testosterone may be acting indirectly on virus-specific $CD8^+$ T cells, through interactions with other cells to promote their contraction. One such cell type may be eosinophils, which accumulated in the lungs of testosterone-depleted males in our model, and

have recently been shown to promote the proliferation and activation of $CD8^+$ T cells following IAV infection in murine models of allergic asthma [217].

The impact of testosterone on infectious disease outcomes involves many cell types and responses. While our work has shown testosterone-induced changes in immune function to be protective, these same immunological changes can be detrimental in other instances, including with amoebic infection, in which treatment with testosterone increases the severity of infection at least in part through inhibition of IFNγ production by natural killer T cells [114, 143, 154]. When disease following infection is caused by the inability to control the pathogen, then androgens, like testosterone, are detrimental. Conversely, when disease following infection is largely attributable to immunopathology [54, 267], the immunomodulatory effects of testosterone are likely protective. These data suggest that testosterone confers protection during IAV infection by modulating the immune response and suggest that testosterone may have therapeutic potential in hypogonadal male populations.

Acknowledgements

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Figure and table legends

Fig. 3.1. Testosterone depletion increases the severity of influenza A virus (IAV) infection. Adult male mice were gonadectomized and implanted with either testosterone (gdx + T) or placebo (gdx) containing capsules or received sham surgeries (intact) prior to inoculation with a sub-lethal dose of ma2009 H1N1 IAV. Plasma and seminal vesicles were collected at 21 days post inoculation (dpi) and testosterone concentrations (A) were analyzed by ELISA, while seminal vesical mass as a percentage of total body mass was calculated as a biomarker of androgenic activity (B; n = 11-12/treatment). Mice (n = 13-15/treatment) were monitored daily for changes in body mass (C), body temperature (D), and clinical disease severity (E). Infectious virus was measured in the lungs by $TCID_{50}$ at 3, 7, and 14 dpi (F; n = 6-9/treatment/time-point). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*$p < 0.05$).

Fig. 3.2. Testosterone does not alter pulmonary cytokine or chemokine concentration during influenza A virus (IAV) infection. Adult male mice were gonadectomized and implanted with either testosterone (gdx + T) or placebo (gdx) containing capsules, and then inoculated with a sub-lethal dose of ma2009 H1N1 IAV or were mock infected. At 0, 3, 9, or 14 dpi (n = 8-11/treatment/time-point), lung tissue was collected and homogenized, and cell free supernatants were used to quantify pulmonary concentrations of IL-6 (A), IL-12p40 (B), G-CSF (C), IFNγ (D), CCL2 (E) and IL-10 (F). Data represent means +/- SEM from two independent replications. No differences were seen between treatment groups.

Fig. 3.3 Testosterone treatment reduces numbers of eosinophils and activity of virus-specific $CD8^+$ T cells following control of viral replication. Adult male mice were gonadectomized and

implanted with either testosterone (gdx + T) or placebo (gdx) containing capsules, and then inoculated with a sub-lethal dose of ma2009 H1N1 IAV or mock infected. At select days post inoculation (dpi), mice were euthanized, and pulmonary immune cells were quantified by flow cytometry (n = 6-14/treatment/time-point). Surface marker and intracellular staining was used to identify numbers of $CD45^+$ cells (A), eosinophils (B), total $CD8^+$ T cells (C), ma2009 H1N1-specific $CD8^+$ T cells (D), $CD8^+$ T cells producing IFNγ (E) in response to *ex vivo* H1N1-specific peptide stimulation, and poly-functional $CD8^+$ T cells expressing both CD107a and IFNγ (F) following *ex vivo* H1N1-specific peptide stimulation (E). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*$p < 0.05$).

Fig. 3.4. The androgen receptor antagonist, flutamide, inhibits the protective effects of testosterone treatment on influenza A virus (IAV) pathogenesis. Adult male mice were gonadectomized and implanted with capsules containing placebo (gdx), testosterone (gdx + T), or flutamide + testosterone (flutamide + T), and seminal vesicle mass was quantified as the percentage of total body mass (A; n = 9/treatment). Following intranasal inoculation with a sub-lethal dose of ma2009 H1N1 IAV, mice were monitored daily for changes in body mass (B) and body temperature (C) for 21 days post inoculation (dpi; n = 12-15/treatment). At 14- and 21-dpi, the total numbers of $CD8^+$ T cells (D), H1N1-specific $CD8^+$ T cell numbers (E), and the number of $CD8^+$ T cells producing IFNγ in response to *ex vivo* H1N1-specific peptide stimulation (F) were quantified by flow cytometry (n = 8-10/treatment/time-point). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*$P < 0.05$).

Fig. 3.5. The non-aromatizable androgen, dihydrotestosterone (DHT) mimics the protective effects of testosterone on influenza A virus (IAV) pathogenesis. Adult male mice were gonadectomized and implanted with capsules containing testosterone (gdx + T), DHT (gdx + DHT), or placebo (gdx), and seminal vesical mass was quantified as the percentage of total body mass (A; n = 6-8/treatment). Following intranasal inoculation with a sub-lethal dose of ma2009 H1N1 IAV, mice were monitored daily for changes in body mass (B) and body temperature (C) for 21 days post inoculation (dpi; n = 13-15/treatment). At 14 and 21 dpi, the total numbers of $CD8^+$ T cells (D), H1N1-specific $CD8^+$ T cell numbers (E), and the number of $CD8^+$ T cells producing IFNγ in response to *ex vivo* H1N1-specific peptide stimulation (F) were quantified by flow cytometry (n = 8-12/treatment/time-point). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*$p < 0.05$).

Fig. 3.6. Testosterone acts indirectly to promote the contraction of $CD8^+$ T cell populations following control of viral replication. Adult male TCR-Ova mice were gonadectomized and implanted with capsules containing testosterone or placebo prior to infection with a sub-lethal dose of WSN-Ova_1 H1N1 IAV (n = 5/treatment group). At 14 days post inoculation (dpi), mice were euthanized, $CD8^+$ T cells were isolated by negative selection, and splenic mRNA was measured and normalized to GAPDH using the ΔCT method (A). Adoptive transfer experiments were preformed, and adult male TCR-Ova and CD90.1 mice were gonadectomized and implanted with capsules containing either placebo (gdx) or testosterone (gdx + T). Splenic $CD8^+$ T cells were isolated from placebo- or testosterone-treated donor TCR-Ova mice by negative selection purification, and adoptively transferred by tail vein injection into either placebo- or testosterone-treated male CD90.1 recipient mice. Mice were then infected by intranasal

inoculation with a sub-lethal dose of WSN-Ova$_1$ H1N1 IAV, and seminal vesicle mass was quantified as the percentage of total body mass (B; n = 6-7/treatment). Mice were monitored daily for changes in body temperature (C). At 14 dpi, mice were euthanized, and lung tissue was collected to quantify the numbers of CD45$^+$ cells (D), eosinophils (E), total CD8$^+$ T cells (F), Ova-specific CD8$^+$ T cells (G), and adoptively transferred CD90.2$^+$ CD8$^+$ T cells producing IFNγ in responses to OVA-specific peptide stimulation (H) were quantified by flow cytometry (n = 6-7/treatment). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*p < 0.05).

Table 3.1. Total numbers of pulmonary myeloid cells following IAV infection in gonadectomized mice treated with placebo (gdx) or testosterone (gdx + T). Data are presented as the mean +/- SEM from 2 independent experiments (n = 8-10/treatment/timepoint) and significant differences between treatment groups are bolded and denoted by asterisks (*P < 0.05).

Table 3.2. Total numbers of pulmonary CD4+ T cells following IAV infection in gonadectomized mice treated with placebo (gdx) or testosterone (gdx + T). Data are presented as the mean +/- SEM from 2 independent experiments (n = 6-12/treatment/timepoint) and significant differences between treatment groups are bolded and denoted by asterisks (*P < 0.05).

Table 3.3. Total numbers of CD8$^+$ T cells in the mediastinal lymph nodes and spleens of gonadectomized mice treated with placebo (gdx) or testosterone (gdx + T) following IAV infection. Data are presented as the mean +/- SEM from 2 independent experiments (n = 5/treatment/timepoint).

Supporting Table 3.1. Pulmonary cytokine and chemokine concentrations (pg/ml) following IAV infection in gonadectomized mice treated with placebo (gdx) or testosterone (gdx + T). Data are presented as the mean +/- SEM from 2 independent experiments (n = 8-11/treatment/timepoint) and significant differences between treatment groups for each timepoint are bolded and denoted by asterisks (*P < 0.05).

Figure 3.1

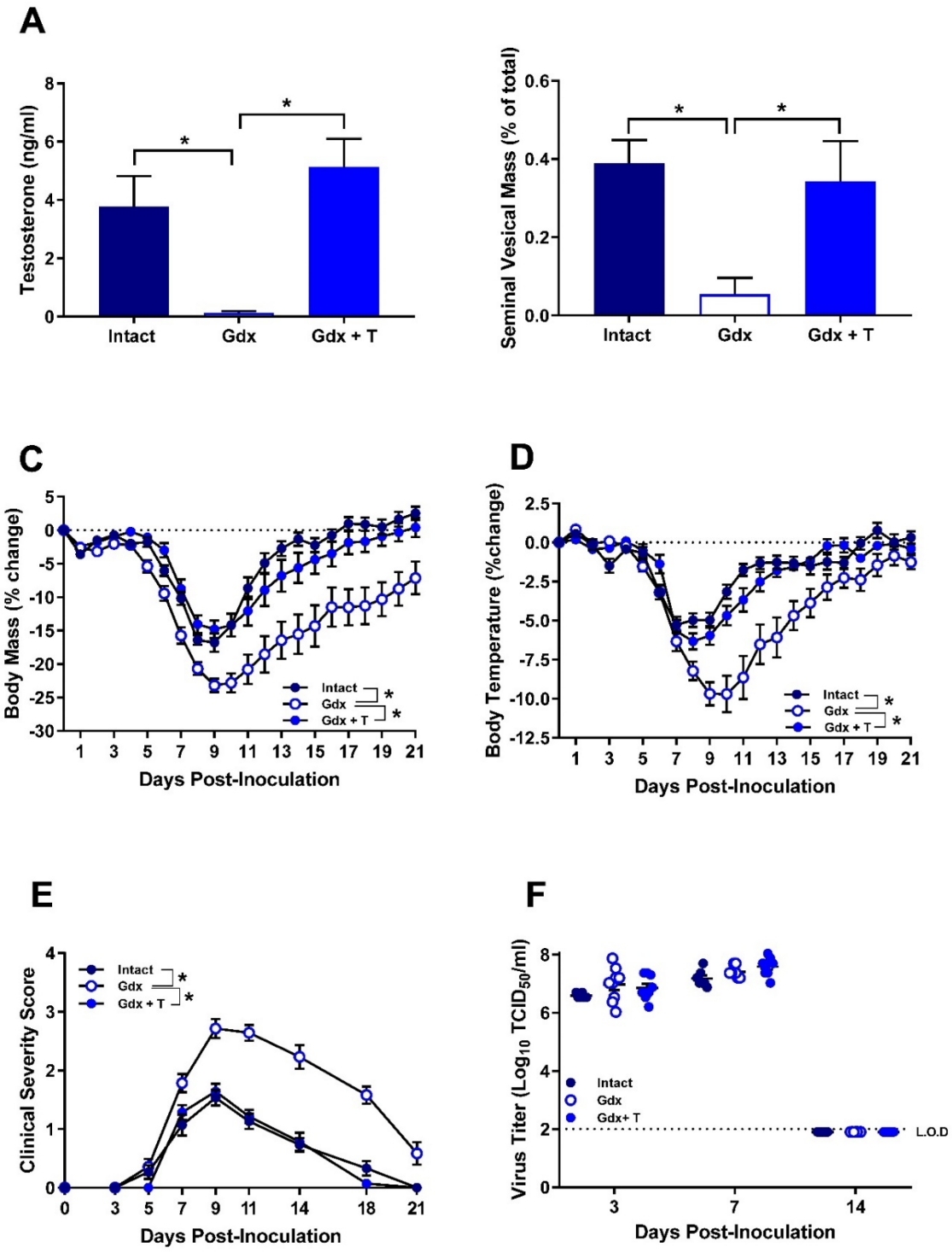
A
Testosterone (ng/ml)
Intact
Gdx
Gdx + T
Seminal Vesical Mass (% of total)
C
Body Mass (% change)
Days Post-Inoculation
Intact
Gdx
Gdx + T
D
Body Temperature (%change)
Days Post-Inoculation
E
Clinical Severity Score
Days Post-Inoculation
F
Virus Titer (Log10 TCID50/ml)
Intact
Gdx
Gdx+ T
L.O.D
Days Post-Inoculation

Figure 3.2

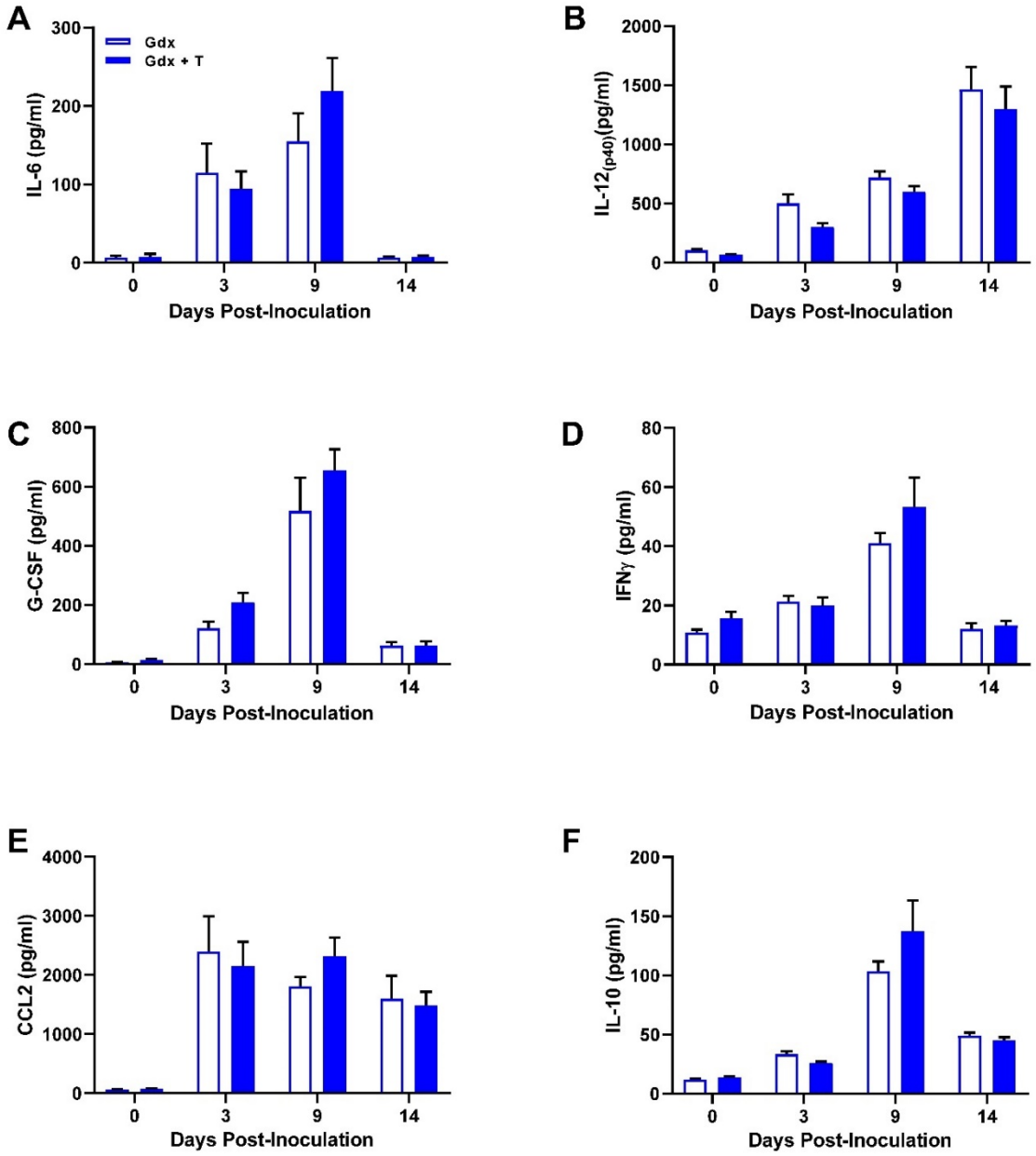
A
Gdx
Gdx + T
IL-6 (pg/ml)
300
200
100
0
0
3
9
14
Days Post-Inoculation
B
IL-12(p40)(pg/ml)
2000
1500
1000
500
0
0
3
9
14
Days Post-Inoculation
C
G-CSF (pg/ml)
800
600
400
200
0
0
3
9
14
Days Post-Inoculation
D
IFNγ (pg/ml)
80
60
40
20
0
0
3
9
14
Days Post-Inoculation
E
CCL2 (pg/ml)
4000
3000
2000
1000
0
0
3
9
14
Days Post-Inoculation
F
IL-10 (pg/ml)
200
150
100
50
0
0
3
9
14
Days Post-Inoculation

Figure 3.3

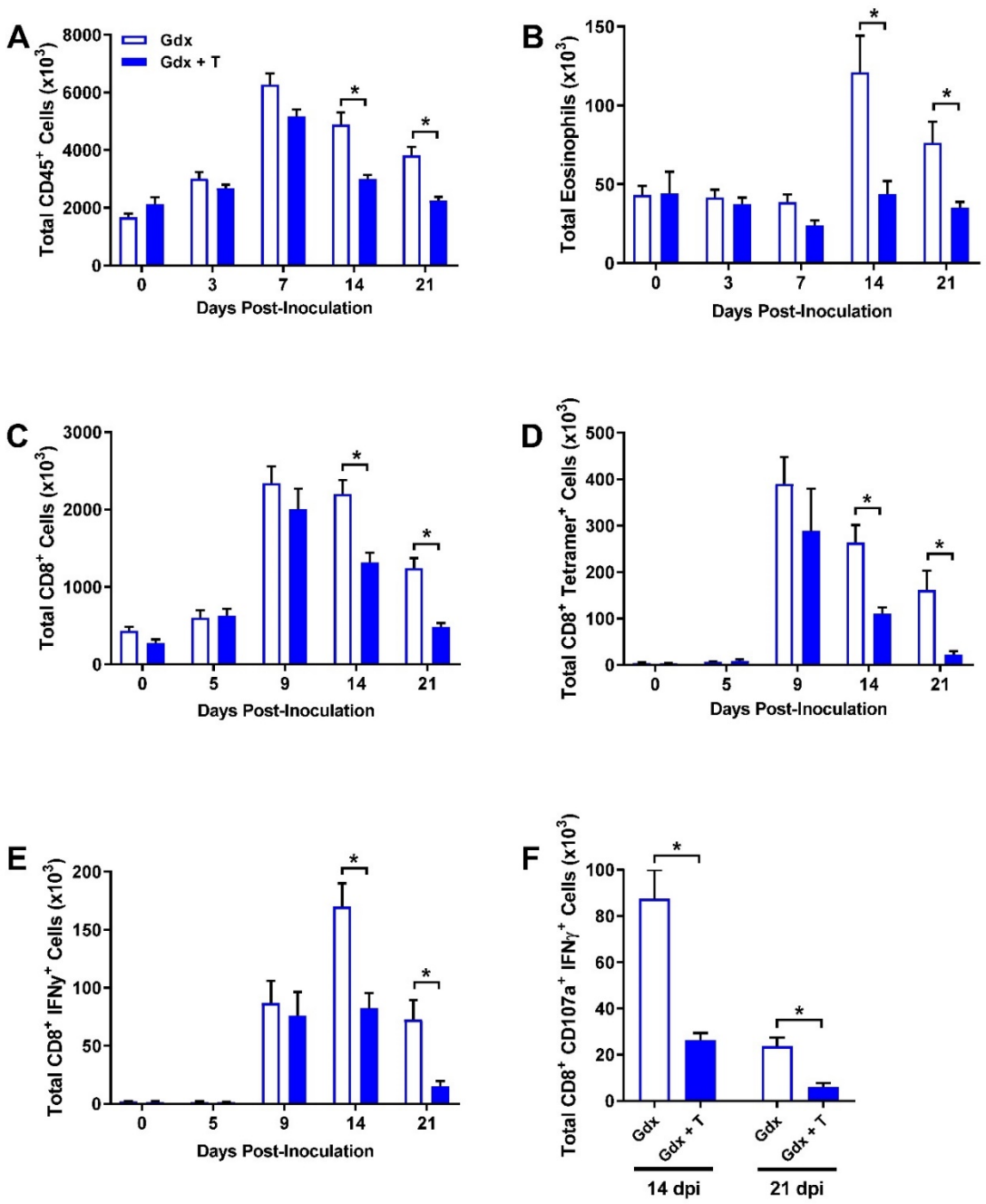
A
Gdx
Gdx + T
Total CD45+ Cells (x10^3)
8000
6000
4000
2000
0
0
3
7
14
21
Days Post-Inoculation
*
*
B
Total Eosinophils (x10^3)
150
100
50
0
0
3
7
14
21
Days Post-Inoculation
*
*
C
Total CD8+ Cells (x10^3)
3000
2000
1000
0
0
5
9
14
21
Days Post-Inoculation
*
*
D
Total CD8+ Tetramer+ Cells (x10^3)
500
400
300
200
100
0
0
5
9
14
21
Days Post-Inoculation
*
*
E
Total CD8+ IFNγ+ Cells (x10^3)
200
150
100
50
0
0
5
9
14
21
Days Post-Inoculation
*
*
F
Total CD8+ CD107a+ IFNγ+ Cells (x10^3)
100
80
60
40
20
0
Gdx
Gdx + T
Gdx
Gdx + T
14 dpi
21 dpi
*
*

Figure 3.4

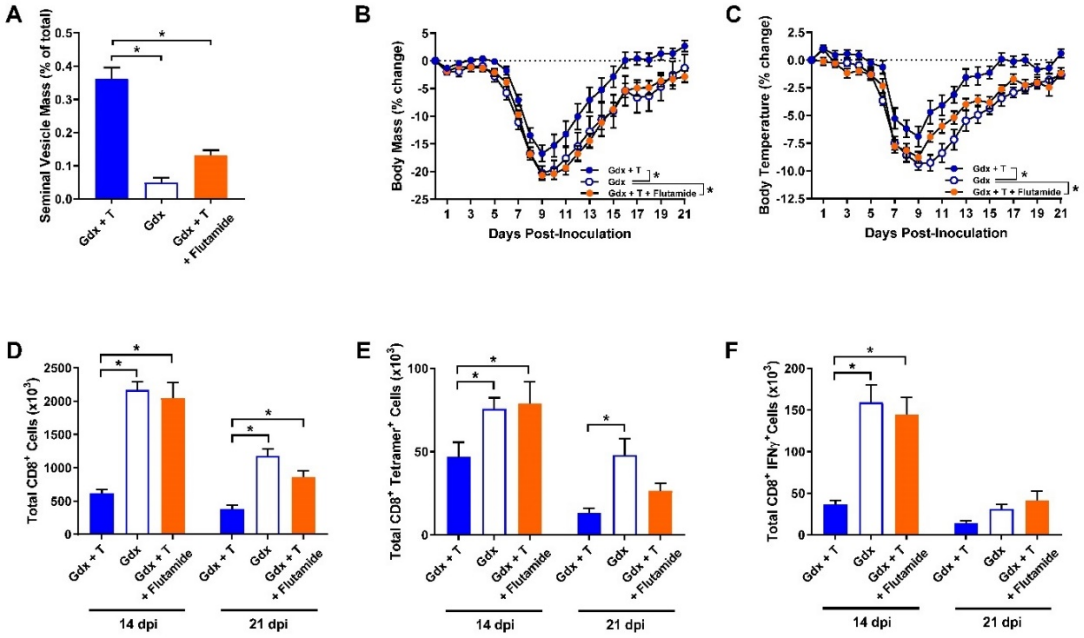
A
Seminal Vesicle Mass (% of total)
Gdx + T
Gdx
Gdx + T + Flutamide
B
Body Mass (% change)
Days Post-Inoculation
C
Body Temperature (% change)
Days Post-Inoculation
D
Total CD8+ Cells (x10-3)
14 dpi
21 dpi
E
Total CD8+ Tetramer+ Cells (x10-3)
F
Total CD8+ IFNγ+ Cells (x10-3)

Figure 3.5

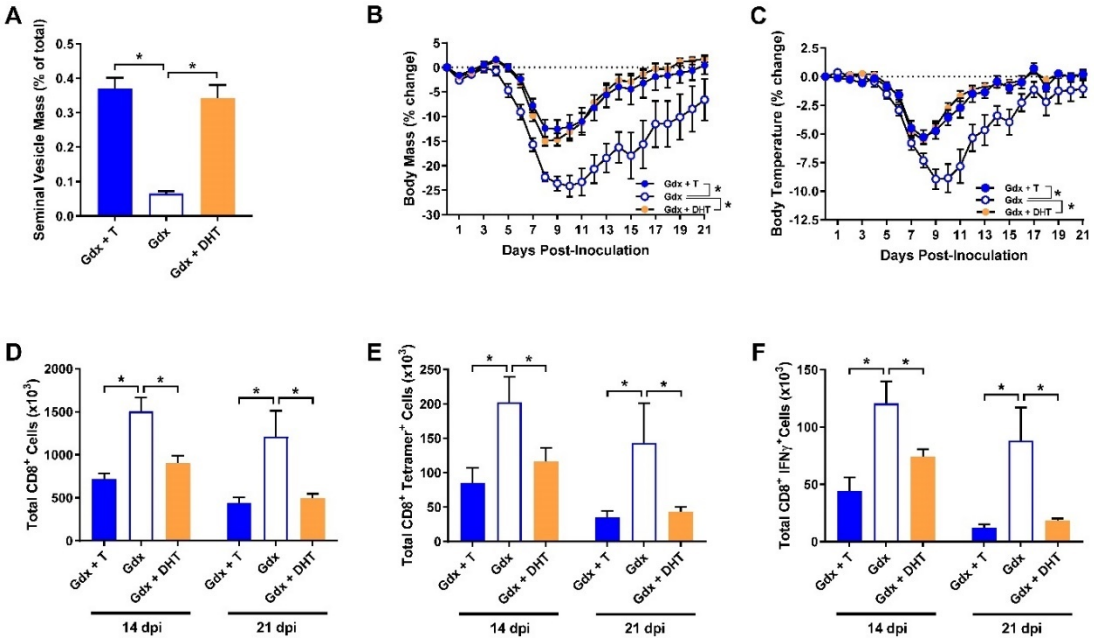
A
Seminal Vesicle Mass (% of total)
Gdx + T
Gdx
Gdx + DHT
B
Body Mass (% change)
Days Post-Inoculation
C
Body Temperature (% change)
Days Post-Inoculation
D
Total CD8+ Cells (x10^3)
14 dpi
21 dpi
E
Total CD8+ Tetramer+ Cells (x10^3)
14 dpi
21 dpi
F
Total CD8+ IFNγ+ Cells (x10^3)
14 dpi
21 dpi

Figure 3.6

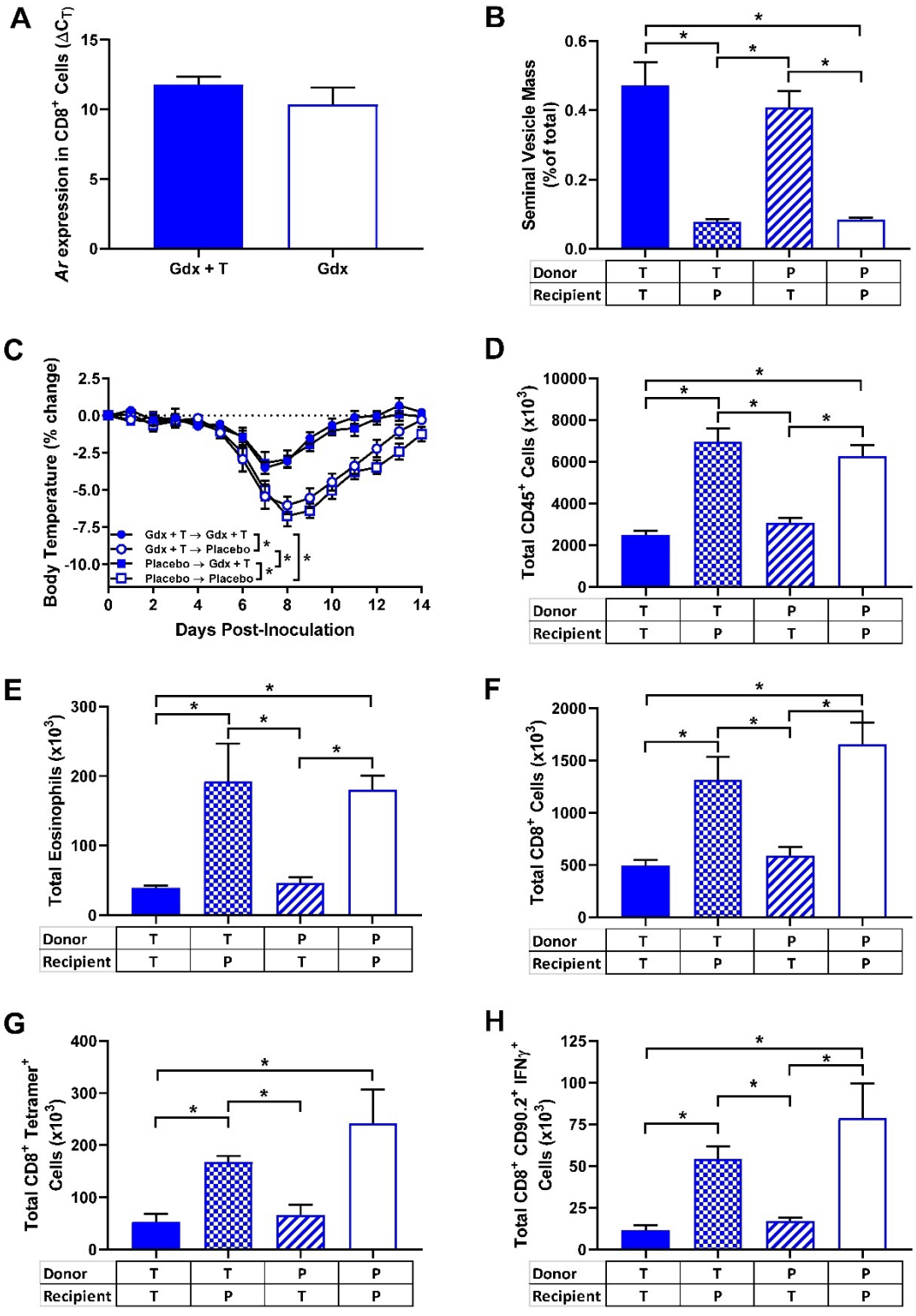
A
Ar expression in CD8+ Cells (ΔCT)
15
10
5
0
Gdx + T
Gdx
B
Seminal Vesicle Mass (%of total)
0.6
0.4
0.2
0.0
Donor T T P P
Recipient T P T P
C
Body Temperature (% change)
2.5
0.0
-2.5
-5.0
-7.5
-10.0
Gdx + T → Gdx + T
Gdx + T → Placebo
Placebo → Gdx + T
Placebo → Placebo
0 2 4 6 8 10 12 14
Days Post-Inoculation
D
Total CD45+ Cells (x10^3)
10000
8000
6000
4000
2000
0
Donor T T P P
Recipient T P T P
E
Total Eosinophils (x10^3)
300
200
100
0
Donor T T P P
Recipient T P T P
F
Total CD8+ Cells (x10^3)
2000
1500
1000
500
0
Donor T T P P
Recipient T P T P
G
Total CD8+ Tetramer+ Cells (x10^3)
400
300
200
100
0
Donor T T P P
Recipient T P T P
H
Total CD8+ CD90.2+ IFNγ+ Cells (x10^3)
125
100
75
50
25
0
Donor T T P P
Recipient T P T P

Table 3.1

	0dpi		3dpi		7dpi		14dpi	
Total numbers of cells	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**
Alveolar macrophages ($x10^3$)	84.4±16.2	97.4±17.5	63.2±8.6	91.2±7.4	72.1±14.7	64.2±15.1	79.4±16.2	72.8±10.3
Interstitial macrophages ($x10^3$)	46.3±6.0	56.7±11.9	188.2±32.1	106.6±9.8	896.0±97.6	812.7±54.2	295.1±38.0	170.3±18.1
Neutrophils ($x10^3$)	**75.5±13.7**	**243.8±40.7***	**260.4±33.4**	**407.7±60.2***	487.0±45.3	487.9±36.6	208.4±45.8	203.4±37.7
Plasmacytoid dendritic cells ($x10^3$)	41.4±8.1	53.7±12.8	170.3±28.9	101.6±12.8	792.5±79.3	672.5±66.3	282.9±34.7	145.7±14.4
Dendritic cells ($x10^3$)	86.6±15.2	90.6±26.1	77.3±8.6	89.4±5.5	241.0±23.5	211.8±13.3	142.2±9.2	105.9±11.8

Table 3.2

	0dpi		5dpi		9dpi		14dpi		21dpi	
Total numbers of cells	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**
Total $CD4^+$ T cells ($x10^3$)	367.1±37.6	289.8±47.3	538.7±61.7	498.8±69.3	1740.5±248.2	1487.7±135.3	**838.5±45.9***	**434.6±39.0**	**611.7±61.7***	**281.1±40.8**
$IFN\gamma^+$ $CD4^+$ T cells ($x10^3$)	3.3±1.2	1.7±0.5	1.3±0.5	3.0±1.8	14.8±3.0	19.1±5.8	7.1±2.7	7.6±1.3	10.4±1.5	7.1±1.4
$IL4^+$ $CD4^+$ T cells ($x10^3$)	6.4±2.0	8.3±1.7	5.3±1.3	6.0±1.7	33.4±5.3	36.3±7.4	24.5±1.3	19.3±2.0	17.3±3.4	8.5±0.9
$IL\text{-}17A^+$ $CD4^+$ T cells ($x10^3$)	4.5±1.5	6.0±1.2	10.2±4.1	6.0±1.7	24.4±2.8	23.0±3.1	7.5±1.3	7.7±1.3	6.0±0.9	3.7±1.0
$CD25^+$ $Foxp3^+$ $CD4^+$ T cells ($x10^3$)	5.3±1.2	3.4±0.8	17.5±2.1	12.5±1.4	28.2±9.6	30.5±7.9	13.8±2.3	12.7±3.6	**16.3±1.4***	**7.3±0.8**

Table 3.3

	Mediastinal Lymph Nodes				Spleen			
	14dpi		21dpi		14dpi		21dpi	
Total numbers of cells	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**
Total $CD8^+$ T cells ($x10^3$)	1687.8±128.6	1442.9±166.5	1425.7±150.8	1388.1±263.8	8030.1±957.2	7949.4±599.9	6413.7±405.8	7943.4±614.6
Tetramer$^+$ $CD8^+$ T cells ($x10^3$)	22.0±6.5	13.3±2.4	12.1±1.4	9.5±1.2	75.1±10.2	94.3±17.9	77.6±17.3	77.2±10.9
$IFN\gamma^+$ $CD8^+$ T cells ($x10^3$)	7.9±1.2	6.7±1.5	6.8±2.0	3.7±1.0	69.2±18.0	70.8±13.0	35.2±8.0	36.2±12.5

Supporting Table 3.1

	0dpi		3dpi		9dpi		14dpi	
Cytokine/Chemokine	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**	**Gdx**	**Gdx + T**
IL-1α	19.2±1.7	37.1±5.0	25.4±1.9	32.2±2.5	23.9±2.2	33.2±6.0	19.0±1.1	15.7±1.3
IL-1β	120.1±6.7	151.9±6.9	122.3±8.6	116.4±5.1	253.0±9.3	269.2±12.8	124.6±7.3	145.8±8.2
IL-2	15.7±1.4	19.0±2.7	13.3±1.1	11.1±0.9	11.2±0.5	13.7±0.5	6.6±0.1	6.3±0.4
IL-3	2.2±0.1	2.9±0.2	3.8±0.4	3.6±0.3	14.2±1.7	12.1±1.3	8.5±2.3	6.1±0.9
IL-4	2.1±0.7	2.4±0.6	3.4±0.4	2.8±0.3	6.1±0.6	6.6±0.4	5.8±0.6	4.3±0.4
IL-5	6.0±0.7	6.4±0.4	14.0±1.9	8.5±0.9	19.1±2.6	27.3±2.8	20.7±2.5	13.9±1.2
IL-6	6.7±2.4	7.6±3.7	114.8±37.1	94.2±22.3	154.8±35.9	219.2±42.2	6.7±1.5	7.4±1.9
IL-10	12.0±0.8	13.6±1.0	33.4±2.6	26.0±1.3	103.5±8.2	137.5±25.9	48.9±2.7	45.1±2.7
IL-12 (p40)	106.2±8.6	70.3±4.3	501.1±76.3	302.8±32.4	719.0±53.6	599.1±46.1	1464.8±190.5	1300.9±189.0
IL-12 (p70)	15.5±1.8	14.6±1.1	58.4±7.2	45.0±3.8	83.8±6.8	87.8±6.2	80.9±7.5	63.8±5.6
IL-13	57.6±9.2	54.9±3.8	155.6±11.0	133.4±4.0	97.7±3.4	108.8±3.4	135.3±5.3	142.0±6.6
Eotaxin	271.5±22.1	286.1±7.9	687.0±70.9	895.3±72.8	267.2±8.8	297.0±18.7	450.9±142.4	423.8±95.3
G-CSF	5.7±1.7	14.7±3.0	120.8±22.6	209.8±31.3	517.5±113.1	655.4±70.8	62.3±12.0	63.1±13.6
GM-CSF	72.6±6.8	69.1±3.7	117.7±8.8	119.3±7.1	61.7±3.6	70.4±2.0	106.5±7.8	96.1±9.1
IFNγ	10.9±0.9	15.6±2.3	21.3±2.0	19.9±2.7	41.1±3.4	53.3±10.0	12.0±2.1	13.2±1.6
CXCL1	**103.7±20.9**	**683.7±344.5***	**959.1±161.9**	**1441.5±124.5***	527.6±50.7	705.5±46.3	**185.4±12.2**	**816.0±271.2***
CCL2	58.4±11.6	75.1±8.7	2395.5±600.2	2150.0±412.5	1811.9±155.3	2316.8±313.2	1599.2±390.2	1485.1±233.5
CCL3	34.8±2.1	31.3±3.9	100.3±17.6	95.1±11.8	1275.3±162.3	1894.1±375.7	277.8±34.1	173.6±27.0
CCL4	28.9±1.0	33.6±1.9	53.2±6.7	42.7±3.5	455.5±43.7	623.4±106.0	61.0±11.2	45.3±5.4
CCL5	304.0±26.0	230.9±26.2	464.2±61.2	375.0±31.0	1007.6±176.3	1121.6±121.1	1222.8±150.9	994.8±130.9
TNFα	81.8±4.8	105.0±7.0	69.4±4.0	62.5±1.8	129.0±3.7	147.2±6.2	62.4±1.7	64.5±3.0
TGFβ	1360.9±222.9	1958.8±383.7	2317.7±328.6	1880.6±127.1	1726.8±259.7	2604.9±405.2	1199.4±128.7	1417.4±160.8

Chapter 4

Irradiated Sporozoite Vaccination Induces Sex-Specific Immune Responses and Protection Against Malaria in Mice

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Abstract

Adult females tend to develop greater adaptive immune responses than males following receipt of either viral or bacterial vaccines, in both preclinical animal studies and human clinical trials. While there is currently no approved malaria vaccine, several anti-sporozoite vaccines, including RTS,S/AS01 and attenuated sporozoite vaccines, are in development, but the impact of sex or age on their efficacy remains undefined. To examine sex differences in the efficacy of anti-sporozoite stage malaria vaccination, adult (10 weeks of age) or juvenile (11 days of age) male and female C3H mice were twice vaccinated with irradiated transgenic *Plasmodium berghei* sporozoites expressing the *P. falciparum* circumsporozoite (CSP) protein and 45 days post boost vaccination mice were challenged with transgenic *P. berghei* via mosquito bite or intradermal challenge. Immunization with irradiated sporozoites resulted in greater protection against challenge in adult females, which was associated with greater anti-CSP antibody production and avidity, as well as greater hepatic, but not splenic, $CD8^+$ T cell IFNy production in adult females relative to adult males. No sex differences in adaptive immune responses or protection were observed in mice vaccinated prior to puberty, suggesting a role for sex steroid hormones. Depletion of testosterone in males increased, whereas rescue of testosterone decreased, anti-CSP antibody production, the number of antigen-specific $CD8^+$ T cells isolated from the liver, and protection following parasite challenge. Conversely, depletion of sex hormones in female mice did not alter vaccine response or protection following challenge. These data suggest that elevated testosterone concentrations in males reduce adaptive immunity and contribute to sex differences malaria vaccine efficacy.

Introduction

Malaria is a significant global burden of disease worldwide, with most cases occurring in sub-Saharan Africa. In 2017, malaria was estimated to result in 216 million clinical cases and 445,000 deaths [332]. Worldwide, malaria causes approximately 5% of all deaths in children under 5 years of age, primarily due to *Plasmodium falciparum* in sub-Saharan Africa [333]. In response, the World Health Organization (WHO) has set out a strategy to achieve a 90% reduction in malaria cases and mortality by the year 2030 [336]. Current methods to interrupt malaria transmission, however, including the early diagnosis and treatment of cases, vector control (e.g., insecticide treated bed net use and residual indoor insecticide spraying), prophylactic drug treatment, and healthcare capacity improvements, are economically costly and unlikely to achieve these goals on their own [337]. To complement these approaches, the need for a malaria vaccine that is at least 75% protective has been identified and would likely be the most cost-effective method for controlling malaria [337, 342].

The RTS,S/AS01 vaccine is currently the leading candidate malaria vaccine undergoing pilot implementation in several regions of sub-Saharan Africa [342]. The RTS,S/AS01 vaccine targets the pre-erythrocytic stage of the malarial life-cycle and consists of the carboxy-terminal region of the *P. falciparum* circumsporozoite (CSP) surface protein along with the hepatitis B surface antigen. In Phase III clinical trials, the RTS,S/AS01 vaccine was shown to be moderately efficacious with 18-36% protection against clinical disease in young boys and girls (i.e., ages 6-12 weeks and 5-17 months) [350]. The RTS,S/AS01 vaccine, however, was also associated with higher all-cause mortality in girls, but not in boys, in both age groups in which the vaccine was tested [393]. Though significant, the mechanisms underlying this sex-differential outcome remain unclear, but these observations highlight the potential for sex-specific differences in the outcomes of malaria vaccination [452]. While no other malaria candidate vaccine has reached

Phase III clinical trials, other candidate vaccines, including several whole sporozoite vaccines, are currently undergoing preliminary clinical trials [343]. Regardless of the candidate vaccine, to date, clinical trials have been conducted solely in healthy adult volunteers or children without consideration of the influence of sex as a biological variable [453].

Clinical data illustrate that in both children and adults, males and females can differ in vaccine-induced immune responses and protection [166]. Following vaccination, juvenile and to a greater extent adult females often develop higher antibody responses, generate more robust cell-mediated immunity, and are better protected by vaccination, but also experience more frequent and severe adverse reactions than age matched males [165, 166, 454]. The mechanisms mediating these vaccine-associated differences are incompletely understood, but have been attributed to the effects of sex steroid signaling, sex chromosome complement, epigenetic regulation, and the microbiome on immune responses to vaccine antigens [166]. Less well known is the influence that the route of administration, vaccine composition (e.g. live attenuated versus inactivated), dose, and pathogen type may have on sex differences in vaccine-induced immune responses. Our current knowledge of the influence of sex on vaccination has generally been informed by vaccines targeting viruses and bacteria [165, 166], with little clinical data pertaining to vaccines against parasitic infections, including malaria.

Preclinical animal models have proven useful for studying vaccine efficacy and the immune response to malaria [455]. But here to, little consideration has been given to sex as a biological variable, with most studies either not reporting the sex of the animals or only using female animals [456-459]. In murine studies of malaria blood stage infection, females have reduced mortality, experience faster resolution of infection associated anemia and weight loss, and mount a more robust immune response to infection (e.g. increased IFNγ, IL-10, and plasmodium specific IgG1 antibody production) than males [380]. Consistent with these

observations, females are better protected against challenge than males following vaccination with the surface membranes of *P. chabaudi* infected red blood cells [168]. To date, no pre-clinical study has been adequately designed or analyzed to study sex differences in the immunogenicity and efficacy of pre-erythrocytic stage malaria vaccination. In this study, we examined the influence of sex, age, and sex hormones on the efficacy and immune response to pre-erythrocytic malaria vaccination using an irradiated sporozoite vaccine model.

Materials and Methods

Mice

All animal procedures were approved by the Johns Hopkins Animal Care and Use Committee (M016H35). Adult (8-10 weeks old) male and female C3H/HeNCr MTV mice were purchased from Charles River (Wilmington, MA) and housed at 5 animals per microisolator cage. For studies using juvenile mice, time pregnant (arrived at embryonic day 12) female C3H/HeNCr MTV mice were purchased from Charles River (Wilmington, MA) and housed as individual dams with pups until weaning. Pups were weaned at post-natal day (PND) 21, separated by sex, and housed at 3-5 animals per microisolator cage. All mice were housed under standard BSL-2 housing conditions and given food and water *ad libitum*.

Irradiated sporozoite vaccination

Previously generated transgenic *P. berghei* sporozoites expressing the immunodominant *P. falciparum* circumsporozoite (CSP) protein (*P.b.-P.f.*) were used for all vaccinations [357]. For studies in adult mice, mice were twice vaccinated at 14-day intervals by tail vein injection with 1 x 10^5 sporozoites gamma irradiated at a dose of 25gy using Cs-137 as the source (GammaCell 1000). Due to the body size and inaccuracy of accessing the tail vein, juvenile mice were twice vaccinated by intraperitoneal injection at 14-day intervals beginning at PND 11 with 1 x 10^5 gamma irradiated *P.b.-P.f*. sporozoites.

Anti-circumsporozoite protein enzyme-linked immunosorbent assays (ELISA)

ELISA plates (Greiner Bio-One) were coated with 100µl per well of purified recombinant CSP peptide [357] diluted in 1X Phosphate buffered Saline (PBS, Gibco) at a concentration 1µg/ml. After incubation overnight at room temperature (RT), plates were washed three times with

200µl of PBS, before blocking with 200µl PBS-BSA (1X PBS with 1% BSA, Sigma) for 2 hours at RT. Following three washes with PBS, serially diluted plasma samples were added, and incubated at RT for 1 hour. The plates were washed two times with PBS-Tween (1X PBS with 0.5% Tween20, Sigma) followed by two washes with PBS and 100µl of secondary antibody (IgG; KPL, IgG1; ThermoScientific, or IgG2a; Invitrogen) at a 1:1000 dilution was added for 1 hour at RT. Plates were then washed three times with PBS-Tween followed by three times with PBS and 100µl of horseradish peroxidase substrate (KPL) was added to each well and developed in the dark for 15minutes. The reaction was stopped using 50µl of 1% SDS (Fisher) and the plates were read at 405nm. Antibody titers were calculated as the highest plasma dilution with an optical density (OD) value greater than three times the average OD of the negative controls. Titer curves were plotted after normalization to the negative control and the area under the titer curve (AUC) was calculated and the dilution titer equal to an optical density of 1 (OD1) was determined by non-linear regression. For semiquantitative assessment of antibody concentration, a titer curve was generated using known quantities of the *P. falciparum* CSP specific monoclonal antibody 2A10 [460], with a concentration of 400µg/ml assigned a value of 1000 ELISA Units (EU). Non-linear regression was then used to generate an equation for the reference antibody curve and the OD values for each experimental sample were interpolated into the reference antibody equation to determine the relative concentration in EUs. For all calculations, only the linear portion of the curve was used and resulting concentrations were multiplied by the dilution factor to obtain the final relative antibody concentration for each experimental sample.

Anti-circumsporozoite (CSP) avidity assay

ELISA plates were coated with of 100µl per well of purified CSP peptide [357] diluted in PBS at a concentration 1µg/ml. After incubation overnight at RT, plates were washed three times with

200µl of PBS, before being blocked with 200µl PBS-BSA for 2hrs at RT. Following three washes with 200µl PBS, plasma samples were plated in quadruplicate at a 1:200 dilution in PBS-BSA and incubated for 1 hour at room temperature. Plates were washed two times with 200µl PBS-Tween followed by two washes with PBS. To measure antibody avidity, 2M ammonium thiocyanate (NH4SCN; Sigma) or PBS was added to the plates for exactly 15 minutes. The plates were washed two times with PBS-Tween followed by two times with PBS and peroxidase-labeled goat anti-mouse IgG antibody was added at a concentration of 500ng/ml and incubated for 1 hour at RT. Plates were washed three times with PBS-Tween followed by three times with PBS and 100µl of horseradish peroxidase substrate was added to each well and developed in the dark for 15minutes. The reaction was stopped using 50µl of 1% SDS and the plates were read at 405nm. The antibody avidity index was determined by dividing the NH4SCN treated optical density values by the corresponding PBS (untreated) values for each sample in duplicate.

Sporozoite challenge

Mice were sedated with Ketamine-Xylazine and challenged by mosquito bite using 10 *P.b.-P.f.* infected female *Anopheles stephensi* mosquitoes or challenged by intradermal injection with 3 x 10^3 chimeric *P.b.-P.f.* CSP sporozoites 45 days after the boost vaccination. Hepatic parasite loads were quantified forty-two hours post-challenge by RT-qPCR targeting *P. berghei* 18s rRNA using forward primer 5' -TGGGAGATTGGTTTTGACGTTTATGT- 3' and reverse primer 5' -AAGCATTAAATAAAGCGAATACATCCTTAC-3' as described previously [461]. Resulting parasite loads were expressed as *P. berghei* 18s rRNA copy number as well as the log and percent reduction relative to naïve controls.

CD8$^+$ T cell responses

Single cells suspensions were generated by homogenizing tissue through a 100µm nylon filter (Falcon) followed by ACK lysis of red blood cells (Quality biologicals) for splenic tissue or Percoll gradient separation (GE) for hepatic tissue. The total numbers of live cells for both splenic and hepatic samples were determined using a hemocytometer and trypan blue (Invitrogen) exclusion, and cells were resuspended at 40 × 10^6 cells/ml in RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (Fisher Scientific), 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). Isolated cells were plated out at 2 x 10^6 cells/well and incubated for 5-6hrs at 37°C with 5 x 10^5 cells/well of either peptide pulsed (DYENDIEKKI – 10ug/10 x 10^6 cells) or non-pulsed LM1 cells at in the presence of GolgiPlug (BD) and GolgiStop (BD). Following incubation, Fc receptors were blocked using anti-CD16/32 (BD Biosciences) and cells were stained with CD8-PerCPCy5.5 (Clone 53-6.7; BD). Cells were then permeabilized and fixed (BD Cytofix/Cytoperm) prior to intracellular staining with IFNγ -FITC (Clone XMG1.2; BD) and TNFa-PE (Clone MP6-XT22; BD). Data were acquired using a FACSCailubr flow cytometer (BD) running Cell Quest Pro and analyzed using FlowJo (v.10) software (Tree Star, Inc.).

Gonadectomy and hormone replacement

Adult male and female mice (9-10 weeks) were bilaterally gonadectomized (gdx) or sham surgeries were performed with the mice remaining gonadally intact as previously described [164]. For males, two weeks following gonadectomy, mice were implanted subcutaneously with silastic tubing capsules (inner diameter-0.04", outer diameter- 0.085"; HelixMark) containing crystalline testosterone propionate (gdx + T; 10.0 mm; Sigma) of left empty (gdx) [433]. The capsules were sealed with 2.5 mm of medical adhesive (Factor II, A-100), and incubated at 37°C

overnight in sterile saline solution prior to implantation. Capsules were replaced prior to depletion every 28 days for the duration of the study.

Sex-hormone enzyme immunosorbent assays

Plasma was collected 3 days prior to challenge and total testosterone, or estradiol concentrations were quantified by commercial EIA kits according to the manufacturer's instructions (IBL America – testosterone; Calbiotech – estradiol). To prevent sample degradation, care was taken to limit light and thermal exposure of plasma samples prior to hormone quantification.

Statistical analysis

Data were analyzed by Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's test for pairwise multiple comparisons. Statistical analyses were performed using GraphPad Prism 7.05 software and mean differences were considered significant at $P < 0.05$.

Results

Adult female mice mount greater antibody responses to irradiated sporozoite vaccination

To test if the sexes respond differently to malaria vaccination, we used an established model where adult male and female C3H mice were twice vaccinated with 1 x 10^5 irradiated *P.b.-P.f.* sporozoites by intravenous injection [462]. Forty-two days post boost vaccination, anti-CSP IgG titers were significantly greater in adult females than males (Mann-Whitney, $P < 0.05$; **Fig. 4.1A**). To confirm these findings, anti-CSP IgG data were further analyzed for the area under the curve (AUC), the titer equal to an optical density-1 (OD1), and antibody quantity. Regardless of the method used to quantitatively measure anti-CSP IgG antibody responses, adult females exhibited greater responses following vaccination than adult males (Mann-Whitney, $P < 0.05$ in each case; **Fig. 4.1B-D**).

In addition to antibody quantity, qualitative differences in humoral responses can influence vaccine efficacy. To assess the impact of sex on qualitative antibody traits following irradiated sporozoite vaccination, antibody avidity, which is associated with protection from sporozoite challenge [457], was measured. Anti-CSP antibody avidity was significantly greater in adult female compared to adult male mice (Mann-Whitney, $P < 0.05$; **Fig. 4.1E**). Because avidity can be influenced by antibody isotype and IgG1, but not IgG2a/c, titers are associated with sterile immunity against *P. berghei* [457], we evaluated sex differences in IgG isotypes. Overall, females had significantly greater anti-CSP IgG1 and IgG2a antibody titers than males (Mann-Whitney, $P < 0.05$ in each case; **Fig. 4.1F** and **Fig. 4.1G**). The IgG1:IgG2a ratio, however, was similar between the sexes (Mann-Whitney**, Fig. 4.1H**), suggesting that no sex-specific shift in Th2/Th1 skewing was apparent. Taken together, these data suggest that the quality and quantity of antibody following receipt of an irradiated sporozoite vaccine is greater in adult female than male mice.

Adult female mice have increased numbers of antigen-specific hepatic $CD8^+$ T cells and are better protected following parasite challenge

To evaluate sex differences in irradiated sporozoite vaccine efficacy, adult male and female mice were challenged by mosquito bite using 10 *P.b.-P.f.* infected female mosquitoes 45 days post boost vaccination. Forty-two hours after challenge, hepatic and splenic $CD8^+$ T cells were isolated and the number of cells producing IFNγ in response to CSP-specific peptide stimulation was quantified. Following CSP peptide stimulation, the number of hepatic $CD8^+$ T cells producing IFNγ was significantly greater in adult females than males (Mann-Whitney, $P < 0.05$; **Fig. 4.2A**). In contrast, while peptide stimulation increased production of IFNγ by splenic $CD8^+$ T cells in vaccinated mice, no sex differences were observed (Mann-Whitney, $P < 0.05$ for peptide stimulation; **Fig. 4.2B**). To assess whether sex differences in immune responses to the vaccine resulted in differential vaccine efficacy, we measured hepatic *P. berghei* 18s rRNA copy number as a surrogate for parasite load 42 hours post challenge. Parasite load was lower in vaccinated mice relative to naïve mice of both sexes; mean parasite load among vaccinated adult females, however, was significantly lower relative to adult males (Kruskal-Wallis, $P < 0.05$; **Fig 4.2C**). To determine whether sex differences in parasite load were reflective of differential mosquito bite numbers, and hence exposure, the number of blood fed mosquitoes (i.e. those with visible midgut blood content) was recorded for each mouse. Though the number challenge mosquito bites varied among individual mice, no sex differences in mosquito bite numbers were observed in either vaccinated or naïve mice (Kruskal-Wallis; **Fig 4.2D**).

Because of the variation in mosquito bite numbers, and presumably challenge dose, challenge experiments were repeated using intradermal inoculation with 3×10^3 *P.b.-P.f.* sporozoites at 45 days post boost vaccination. Consistent with mosquito bite challenge, vaccinated mice had greater numbers of $IFN\gamma^+$ $CD8^+$ T cells in the liver and spleen following *ex*

vivo peptide simulation (Mann-Whitney, $P < 0.05$ in each case; **Fig. 4.2E** and **Fig. 4.2F**). Following CSP-specific peptide stimulation, numbers of $CD8^+$ T cells producing IFNy in the liver, but not the spleen, were greater in vaccinated female than male mice following intradermal challenge (Mann-Whitney, $P < 0.05$ for hepatic $CD8^+$ T cells and $P >$ for splenic $CD8^+$ T cells; **Fig. 4.2E** and **Fig. 4.2F**). Also, consistent with mosquito bite challenge, hepatic *P. berghei* 18s rRNA copy number was significantly reduced in both sexes following vaccination, with the magnitude of the reduction being greater in adult females than males (Kruskal-Wallis, $P < 0.05$; **Fig 4.2G**). As another way to analyze the change in parasite load following vaccination, we calculated the log reduction in parasite load relative to the average parasite load in naïve mice for each sex and experimental replication. Consistent with the relative parasite load (**Fig 4.2G**), as compared with naïve mice, the log reduction in parasite load was greater for vaccinated females than males (Mann-Whitney, $P > 0.05$; **Fig. 4.2H**). In summary, these data demonstrate that irradiated sporozoite vaccine efficacy is greater in adult females than in male mice.

Prior to puberty, vaccine responses and efficacy do not differ between the sex

Sex differences in the immune responses to vaccines are often not evaluated in studies of childhood vaccines [166], but there are some vaccines, including the RTS,S vaccine, that are primarily or exclusively administered prior to puberty [342, 463]. To characterize the impact of sex on irradiated sporozoite vaccine response and efficacy prior to puberty, juvenile mice were twice vaccinated by intraperitoneal injection beginning at postnatal day (PND) 11. Forty-two days post boost vaccination, antibody responses were analyzed by measuring anti-CSP IgG titers, AUC, the OD1 titer, and antibody quantity. Although vaccination resulted in detectable antibody responses against CSP, no sex differences in any measure of the antibody response to CSP were detected among mice vaccinated as juveniles (Mann-Whitney, $P > 0.05$**; Fig. 4.3A** and **Suppl. Fig.**

4.1). The avidity of anti-CSP IgG also did not differ between males and female vaccinated as juveniles (Mann-Whitney, $P > 0.05$; **Fig. 4.3B**). Finally, the titers of anti-CSP IgG1 and IgG2a were quantified and neither titers of IgG isotypes nor the ratio of IgG1:IgG2a differed between sexes among mice vaccinated as juveniles (Mann-Whitney, $P > 0.05$; **Fig. 4.3C-E**).

As adults, mice that were vaccinated with irradiated sporozoites as juveniles received an intradermal challenge with transgenic *P.b.-P.f.* parasites. Unlike mice that were both vaccinated and challenged as adults, among mice vaccinated as juveniles and challenged as adults there was no significant increase in the number of hepatic or splenic $CD8^+$ T cells producing IFNγ in response to CSP specific peptide stimulation, which also did not differ between the sexes (Kruskal-Wallis, $P > 0.05$; **Fig. 4.3F** and **Fig. 4.3G**). Although juvenile vaccination was efficacious, there was no sex difference in the log reduction in parasite load (Mann-Whitney; **Fig. 4.3H**). Taken together, these data suggest vaccination prior to puberty does not result in sex differences in vaccine efficacy, suggesting that sex steroid hormones may be involved.

Removal of ovaries has no effect on vaccine-induced immune responses or protection among adult females

To begin to test whether sex hormones in females contribute to greater vaccine-induced immune responses and protection, a subset of females had their ovaries removed thereby reducing concentrations of 17β-estradiol (Mann-Whitney, $P < 0.05$ **Fig. 4.4A**). Removal of the ovaries in adult female mice, however, did not significantly alter either the quality or quantity of anti-CSP IgG at 42 days post boost vaccination as compared with gonad-intact (i.e., sham) female mice (Mann-Whitney, $P > 0.05$; **Fig. 4.4B-E** and **Suppl. Fig. 4.2**). Following sporozoite challenge, while peptide stimulation increased production of IFNγ by both splenic and hepatic $CD8^+$ T cells from vaccinated mice, gonadectomy increased CSP-specific splenic, but not hepatic,

$CD8^+$ T cell production of IFNγ following peptide stimulation (Kruskal-Wallis, $P < 0.05$ for splenic $CD8^+$ T cells and $P > 0.05$ for hepatic $CD8^+$ T cells; **Fig. 4.4F** and **Fig. 4.4G**). Finally, the log reduction in hepatic parasite load following intradermal challenge did not differ between intact or gonadectomized female mice (Mann-Whitney, $P > 0.05$; **Fig. 4.4H**). Taken together these data suggest that immunity and protection from parasite challenge in female mice may be independent of female gonadal sex hormones.

Testosterone suppresses vaccine-induced immune responses and protection in male mice

To determine whether testosterone in males affected irradiated sporozoite vaccine responses, adult male mice underwent sham surgeries or gonadectomy, with half the gonadectomized males receiving endogenous testosterone, which increased circulating testosterone to within the physiological range of gonad-intact males (Kruskal-Wallis, $P < 0.05$; **Fig. 4.5A**). Following vaccination, depletion of testosterone significantly increased, whereas rescue of testosterone significantly decreased, anti-CSP IgG antibody titers and avidity as well as anti-CSP IgG1 antibody titers (Kruskal-Wallis, $P < 0.05$; **Fig. 4.5B**-**F** and **Suppl. Fig. 4.3**). Rescue of testosterone significantly reduced IgG2a antibody titers and the ratio of IgG1:IgG2a relative to testosterone-depleted males (Kruskal-Wallis; **Fig. 4.5E** and **Fig. 4.5F**).

Following intradermal challenge, the depletion of testosterone significantly increased the number of hepatic $CD8^+$ T cells producing IFNγ in response to CSP-specific peptide stimulation, while the rescue of testosterone levels reduced hepatic $CD8^+$ T cell production of IFNγ to a level comparable with gonad-intact male mice (Kruskal-Wallis, $P < 0.05$; **Fig. 4.5G**). Depletion of testosterone also significantly increased the log reduction in parasite load, whereas the rescue of testosterone significantly reduced the log reduction in parasite load as compared

with naïve mice from the same hormone treatment group (Kruskal-Wallis, $P < 0.05$; **Fig. 4.5H**). Taken together, these data suggest that the decreased efficacy of irradiated sporozoite vaccination in adult males may be mediated by testosterone.

Discussion

Sex differences in vaccine-induced immune responses are documented primarily for vaccines that protect against viruses and bacteria [57, 166], with considerably less attention paid to parasitic vaccines, including those that could protect against malaria. In the present study, following irradiated sporozoite vaccination, adult female mice were better protected against sporozoite challenge than age-matched males, with these differences in protection being associated with greater adaptive immune responses, including malaria-specific antibody and $CD8^+$ T cell responses in females compared with males. These findings are consistent with both human and murine studies showing greater immunogenicity and efficacy of erythrocytic stage malaria vaccination in adult females relative to males [168, 348], as well as in murine models of *Plasmodium* infection [380]. Sex differences in sporozoite vaccine-induced immunity and protection were not observed among mice that were vaccinated prior to puberty. The surge of testosterone during puberty in males as opposed to hormonal changes following puberty in females appeared to be a critical mediator of sex differences in vaccine-induced immunity and protection in adults. Manipulation of sex steroid hormones (i.e., estradiol and progesterone) in females had no impact on vaccine-induced immunity and protection, whereas manipulation of testosterone in males dramatically affected the outcome of vaccination.

Malaria vaccination programs will largely target infants and children as these groups suffer the greatest burden of disease from malaria [333, 342, 463]. Sex differences have been described for some infant and childhood vaccines, with females generally, but not always, mounting greater antibody responses to vaccination [166]. In the current study, males and females that were vaccinated as juveniles had similar CSP-specific antibody and CD8+ T cell responses as well as protection against challenge infection. We did not compare vaccine-induced immune responses and protection between adults and juveniles because while adult

mice were vaccinated via intravenous inoculation of irradiated sporozoites, this route of vaccination was not feasible in juvenile mice due to the small size of their tail vein contributing to technical inaccuracies. For juvenile mice, intraperitoneal inoculations were used, which has been shown to be less efficacious for whole sporozoite vaccination [464]. Because the route of vaccination differed between adult and juvenile mice, it is not possible to conclude that the vaccine was less efficacious in juvenile than adult mice, only that the sex difference in vaccine-induced immunity was present in adult, but not juvenile, mice. We cannot, however, rule out that the route of vaccination may impact sex differences in vaccine-induced immunity.

The observation that sex influences the immunogenicity and efficacy of irradiated sporozoite vaccination in adults, but not in mice vaccinated as juveniles, led us to hypothesize that changes in sex hormone concentrations associated with puberty may be involved. At puberty, the ovaries of females begin synthesizing and releasing elevated concentrations of estradiol and progesterone, whereas the testes of males begin synthesizing greater concentrations of testosterone. These sex steroid hormones affect adaptive immune responses both directly by binding to sex steroid receptors on B and T cells as well as indirectly by altering the activity of innate immune cells [130, 465].

In females, estradiol, in particular, can enhance antibody responses to vaccines and infections [56, 166, 380, 466, 467]. In murine models of malaria blood stage infection, treatment of female mice with estradiol alone or in combination with progesterone increased *Plasmodium*-specific IgG1 antibody production as well as IFNy production by splenocytes relative to sex-hormone depleted females [466]. Treatment of female mice with other forms of estrogen, e.g., estriol, or progesterone stimulates antibody responses to influenza virus antigens, whereas progesterone suppresses memory $CD8^+$ T cell formation, at least in response to a primary infection [431, 467, 468]. In the current study, depletion of sex steroids in females did not alter

either the adaptive immune response to vaccination or protection following parasite challenge. Whether these discordant effects of female sex hormone depletion represent differences in the context of vaccination versus infection, the route of inoculation, or differences in the pathways of immune activation warrant future study. Moreover, there may be other sex-specific factors (e.g., genetic or epigenetic factors) that could be contributing to the more robust immune response in adult females relative to adult males.

The immunosuppressive properties of testosterone are well characterized, but few studies have evaluated the impacts of testosterone on the immune response to vaccination. Notably, in humans, elevated serum concentrations are associated with reduced humoral responses to the trivalent inactivated influenza vaccine [104]. To determine if testosterone suppressed the immune response to irradiated sporozoite vaccination in males, testosterone concentrations were depleted by castration and replaced in castrated males with exogenous continuous-release capsules that increased testosterone to within physiological range of adult males. Depletion of testosterone in males increased, whereas the testosterone treatment of castrated males decreased, parasite load following sporozoite challenge. These findings agree with previous murine studies demonstrating that testosterone suppresses resistance to blood stage infection and reduces protection against challenge following erythrocytic stage vaccination [168, 390]. Moreover, immunity against *Plasmodium* sporozoite infection has been shown to be dependent on high concentrations of anti-CSP antibodies and CSP-specific $CD8^+$ T cells [469, 470]. Consistent with these observations, testosterone depletion increased, while testosterone treatment decreased, the magnitude of the CSP-specific antibody response and number of CSP-specific $CD8^+$ T cells isolated from the liver as revealed by IFNγ production following CSP-specific peptide stimulation. In addition to altering the quantity of vaccine

induced antibody produced, testosterone altered the quality of antibody produced in response to vaccination.

Anti-CSP IgG1 antibodies, which are associated with a Th2 cellular responses and parallel antibody avidity, play and important role in sterile immunity against sporozoite challenge [457, 471]. In the current study, depletion of testosterone increased IgG1, but not Th1 associated IgG2a, titers relative to testosterone-replete males, resulting in a skewing of the anti-sporozoite IgG1/IgG2a ratio. Whether testosterone induced changes in $CD4^+$ T helper cell polarization was not evaluated but should be considered in future studies. The precise mechanisms by which testosterone affects antibody and T cell responses to vaccination remains to be determined, and future study of testosterone signaling in B cells and T cells is warranted. Most pre-clinical malaria vaccine studies use exclusively female mice or do not consider the sex of the animals used [456]. In this study, we demonstrate that testosterone in males is a significant factor mediating sex differences in the immunogenicity and efficacy of a preclinical, pre-erythrocytic malaria vaccine design. Greater consideration of biological sex and sex-specific factors are needed in the experimental design and analysis of preclinical animal studies as well as clinical trials of malaria vaccines. Though testosterone is unlikely to influence the outcomes of childhood malaria vaccination, understanding the mechanisms by which testosterone suppresses vaccine efficacy may provide insight leading to improvements in malaria vaccine design.

Most pre-clinical malaria vaccine studies use exclusively female mice or do not consider the sex of the animals used [456]. In this study, we demonstrate that testosterone in males is a significant factor mediating sex differences in the immunogenicity and efficacy of a preclinical, pre-erythrocytic malaria vaccine design. Greater consideration of biological sex and sex-specific factors are needed in the experimental design and analysis of preclinical animal studies as well

as clinical trials of malaria vaccines. Though testosterone is unlikely to influence the outcomes of childhood malaria vaccination, understanding the mechanisms by which testosterone suppresses vaccine efficacy may provide insight leading to improvements in malaria vaccine design and implementation strategies.

Acknowledgments

The authors would like to thank the Pekosz, Zavala, and Davis laboratories for discussions about these data and the Johns Hopkins Malaria Research Institute Parasite and Mosquito Cores as well as the Johns Hopkins BD Immune Function Core.

Figure legends

Fig. 4.1. Effects of sex on antibody responses to irradiated sporozoite vaccination. Adult (8-10 weeks) male and female mice were twice vaccinated with irradiated sporozoites at 14-day intervals. Forty-two days post boost vaccination, plasma was collected and anti-CSP IgG titers (A), areas under the curve (AUC) (B), anti-CSP titers equal to an optical density-1 (C), anti-CSP antibody concentrations (D), anti-CSP antibody avidity (E), anti-CSP IgG1 titers (F), anti-CSP IgG2a titers (G), and the ratio of IgG1/IgG2a (H) were measured. Data represent means +/- the SEM from two independent replications (n = 34-39/sex) and significant differences are denoted with asterisks (*P < 0.05).

Fig. 4.2. Effects of sex on $CD8^+$ T cell responses and protection from sporozoite challenge**.** Naïve and irradiated sporozoite vaccinated adult (8-10 weeks) male and female mice were challenged by mosquito bite 45 days post boost vaccination. The numbers of hepatic (n = 3 pools of 3/sex/treatment) (A) and splenic (n = 16-18/sex/treatment) (B) $CD8^+$ T cells producing IFNγ in response to CSP-specific peptide stimulation were measured 42 hours post challenge. Hepatic *P. berghei* 18s rRNA copy number (C) was measured (n = 9-15/sex/treatment) in naïve and vaccinated male and female mice. The number of blood fed mosquitos was recorded for each challenge (n = 18-20 mice/sex/treatment) (D). Challenge experiments were repeated with intradermal inoculation, and the numbers of hepatic (n = 6 pools of 3/sex/treatment) (E) and splenic (n = 16-18/sex/treatment) (F) $CD8^+$ T cells producing IFNγ in response to CSP peptide stimulation were measured 42 hours post challenge. Hepatic *P. berghei* 18s rRNA copy number was measured (n = 15/sex/treatment) (G) and the log reduction (H) in parasite load relative to naïve controls was determined (n = 15/sex). Data represent means +/- the SEM from two independent replications and significant differences are denoted with asterisks (*P < 0.05).

Fig. 4.3. Effects of sex on vaccine-induced immunity and protection in juvenile mice. Pre-pubertal (2 weeks of age) male and female mice were twice vaccinated with irradiated sporozoites. Forty-two days post boost vaccination, plasma was collected and anti-CSP IgG concentrations (A), anti-CSP antibody avidity (B), anti-CSP IgG1 titers (C), anti-CSP IgG2a titers (D), and the ratio of IgG1/IgG2a (E) were measured (n = 15-18/sex). Naïve and vaccinated juvenile mice were challenged as adults by intradermal inoculation 45 days post boost vaccination and the numbers of hepatic (n = 4-5 pools of 3/sex) (F) and splenic (n = 11-14/sex) (G) $CD8^+$ T cells producing IFNγ in response to CSP peptide stimulation were measured 42 hours post challenge. The log reduction (H) in parasite load relative to naïve controls was determined (n = 9-12/sex). Data represents means +/- SEM from two independent replications. No differences by sex were observed in mice vaccinated as juveniles.

Fig. 4.4. Effects of ovariectomy on irradiated sporozoite vaccination in adult female mice. Adult (8-10 weeks) female mice were assigned to remain intact (i.e., receive a sham surgery) or be ovariectomized (ovx) two weeks prior to being twice vaccinated with irradiated sporozoites. Plasma was collected 42 days post boost vaccination and estrogen (A) (n = 8-9/treatment), anti-CSP IgG antibody concentrations (B), anti-CSP antibody avidity (C), anti-CSP IgG1 titers (D), and anti-CSP IgG2a titers (E) were measured (n = 15-18/ treatment). Mice were challenged by intradermal inoculation and the numbers of hepatic (n = 4 pools of 3/treatment) (F) and splenic (n = 9-10/treatment) (G) $CD8^+$ T cells producing IFNγ in response to CSP-specific peptide stimulation were quantified. The log reduction in parasite load was measured (n = 9-10/treatment) (H) relative to naïve female mice. Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*$P < 0.05$).

Fig. 4.5. Effects of testosterone on irradiated sporozoite vaccination in adult males. Adult (8-10 weeks) male mice were gonadectomized and implanted with placebo (gdx) or testosterone (gdx + T) capsules or received sham surgeries (sham). Mice were twice vaccinated with irradiated sporozoites and 42 days post boost vaccination, plasma was collected and testosterone concentrations (A) were quantified (n = 8-9/treatment). Anti-CSP IgG antibody concentrations (B), anti-CSP antibody avidity (C), anti-CSP IgG1 titers (D), anti-CSP IgG2a titers (E), and the ratio of IgG1/IgG2a titers were measured (n = 18-29/ treatment group). Mice were challenged by intradermal inoculation and the numbers of hepatic (n = 4-8 pools of 3/treatment) (G) $CD8^+$ T cells producing IFNy in response to CSP-specific peptide stimulation were quantified. The log reduction in parasite load (n = 8-15/treatment) (H) relative to naïve male mice was measured 42 hours post challenge. Data represents means +/- SEM from two independent replications and significant differences across treatment groups are denoted by asterisks (*P < 0.05).

Supplemental Fig. 4.1. Juvenile mice were twice vaccinated with irradiated sporozoites beginning at postnatal day 11. Forty-two days post boost vaccination, plasma was collected and anti-CSP IgG titers (A), areas under the curve (AUC) (B), and the anti-CSP IgG titers equal to an optical density-1 were measured (n = 16-18/sex). Data represents means +/- SEM from two independent replications. No differences by sex were observed in juvenile mice.

Supplemental Fig. 4.2. Adult (8-10 weeks) female mice were ovariectomized (ovx) or received sham surgeries (sham) and mice were twice vaccinated with irradiated sporozoites. Plasma was collected 42 days post boost vaccination and anti-CSP IgG titers (A), areas under the curve (AUC) (B), and the anti-CSP IgG titers equal to an optical density-1 were measured (n = 16-

19/treatment). Data represents means +/- SEM from two independent replications. No differences by treatment group were observed.

Supplemental Fig. 4.3. Adult (8-10 weeks) male mice were gonadectomized and implanted with placebo (gdx) or testosterone (gdx + T) capsules or received sham surgeries (sham). Mice were twice vaccinated with irradiated sporozoites and 42 days post boost vaccination, plasma was collected and anti-CSP IgG titers (A), areas under the curve (AUC) (B), and the anti-CSP IgG titers equal to an optical density-1 were measured (n = 17-28/treatment). Data represents means +/- SEM from two independent replications and significant differences across treatment groups are denoted by asterisks (*P < 0.05).

Figure 4.1

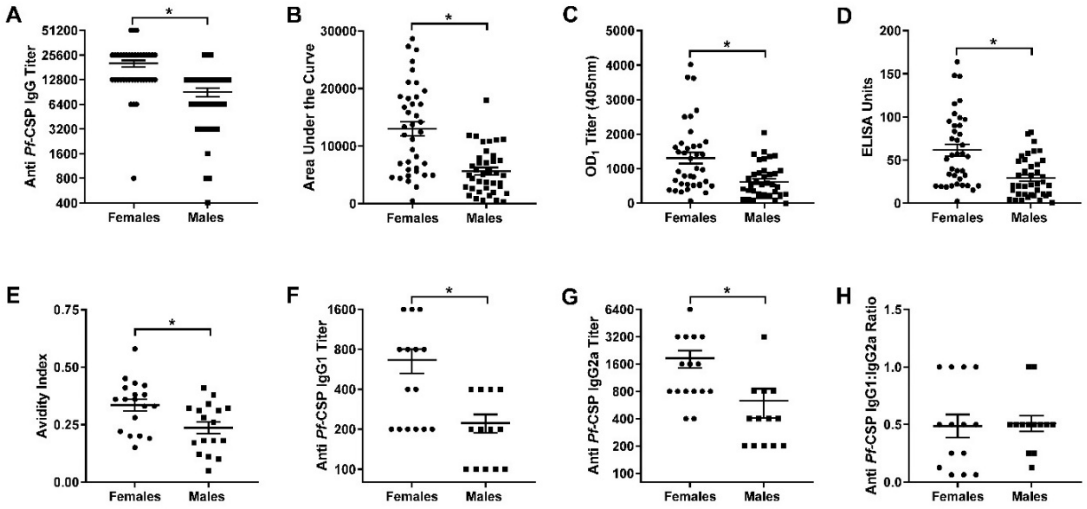

A
Anti Pf-CSP IgG Titer
51200
25600
12800
6400
3200
1600
800
400
Females
Males
*
B
Area Under the Curve
30000
20000
10000
0
Females
Males
*
C
OD1 Titer (405nm)
5000
4000
3000
2000
1000
0
Females
Males
*
D
ELISA Units
200
150
100
50
0
Females
Males
*
E
Avidity Index
0.75
0.50
0.25
0.00
Females
Males
*
F
Anti Pf-CSP IgG1 Titer
1600
800
400
200
100
Females
Males
*
G
Anti Pf-CSP IgG2a Titer
6400
3200
1600
800
400
200
100
Females
Males
*
H
Anti Pf-CSP IgG1:IgG2a Ratio
1.5
1.0
0.5
0.0
Females
Males

Figure 4.2

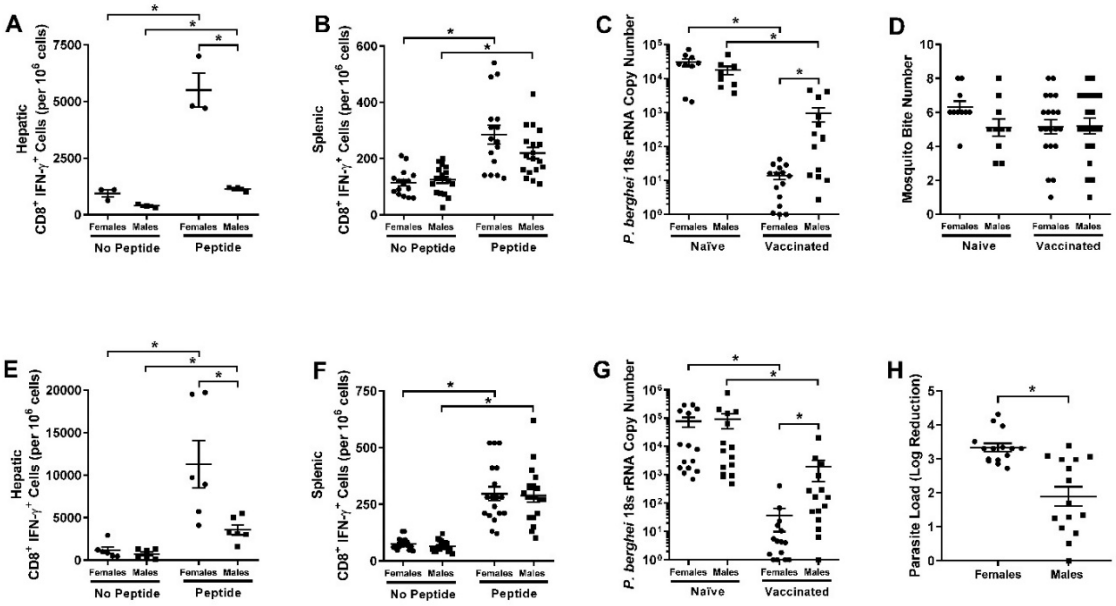

A
Hepatic CD8+ IFN-γ+ Cells (per 10^6 cells)
7500
5000
2500
0
Females Males Females Males
No Peptide
Peptide
B
Splenic CD8+ IFN-γ+ Cells (per 10^6 cells)
600
400
200
0
Females Males Females Males
No Peptide
Peptide
C
P. berghei 18s rRNA Copy Number
10^5
10^4
10^3
10^2
10^1
10^0
Females Males Females Males
Naïve
Vaccinated
D
Mosquito Bite Number
10
8
6
4
2
0
Females Males Females Males
Naive
Vaccinated
E
Hepatic CD8+ IFN-γ+ Cells (per 10^6 cells)
20000
15000
10000
5000
0
Females Males Females Males
No Peptide
Peptide
F
Splenic CD8+ IFN-γ+ Cells (per 10^6 cells)
750
500
250
0
Females Males Females Males
No Peptide
Peptide
G
P. berghei 18s rRNA Copy Number
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Females Males Females Males
Naïve
Vaccinated
H
Parasite Load (Log Reduction)
5
4
3
2
1
0
Females
Males

Figure 4.3

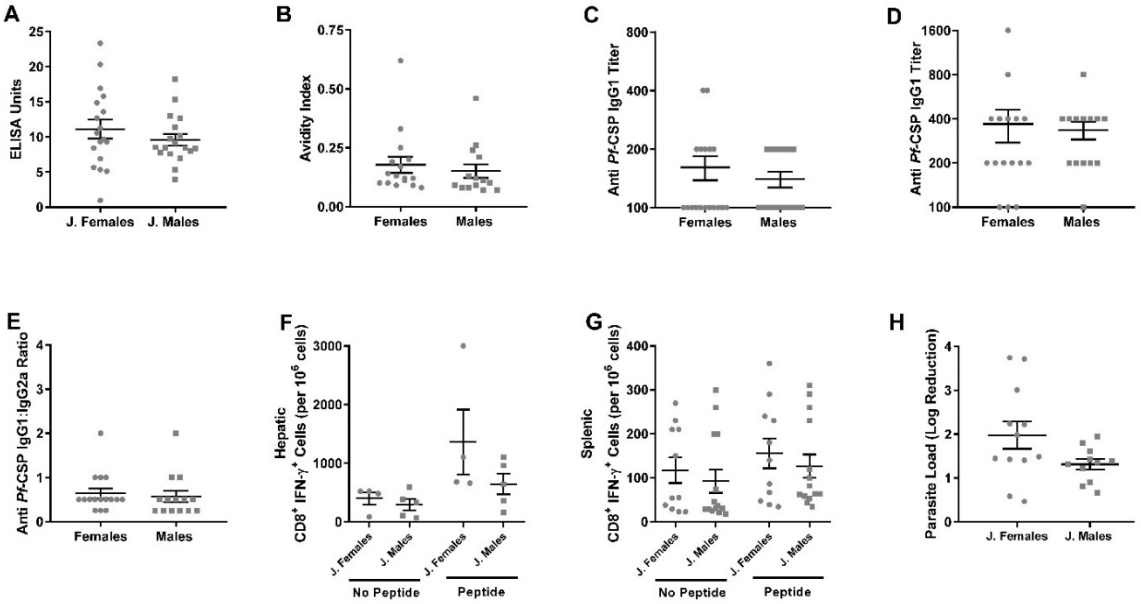

A
ELISA Units
J. Females
J. Males
B
Avidity Index
Females
Males
C
Anti Pf-CSP IgG1 Titer
Females
Males
D
Anti Pf-CSP IgG1 Titer
Females
Males
E
Anti Pf-CSP IgG1:IgG2a Ratio
Females
Males
F
Hepatic
CD8+ IFN-γ+ Cells (per 10^6 cells)
J. Females
J. Males
J. Females
J. Males
No Peptide
Peptide
G
Splenic
CD8+ IFN-γ+ Cells (per 10^6 cells)
J. Females
J. Males
J. Females
J. Males
No Peptide
Peptide
H
Parasite Load (Log Reduction)
J. Females
J. Males

Figure 4.4

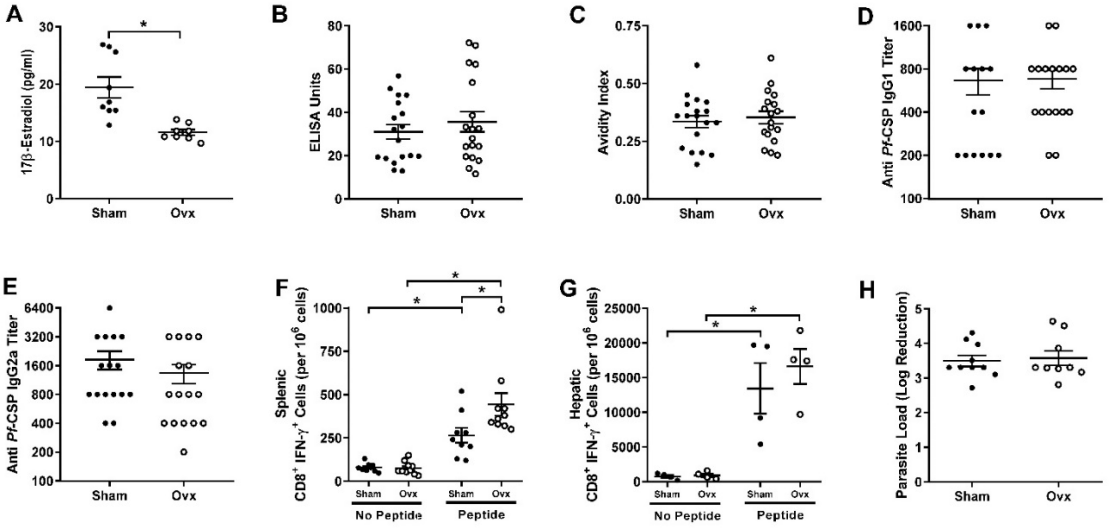
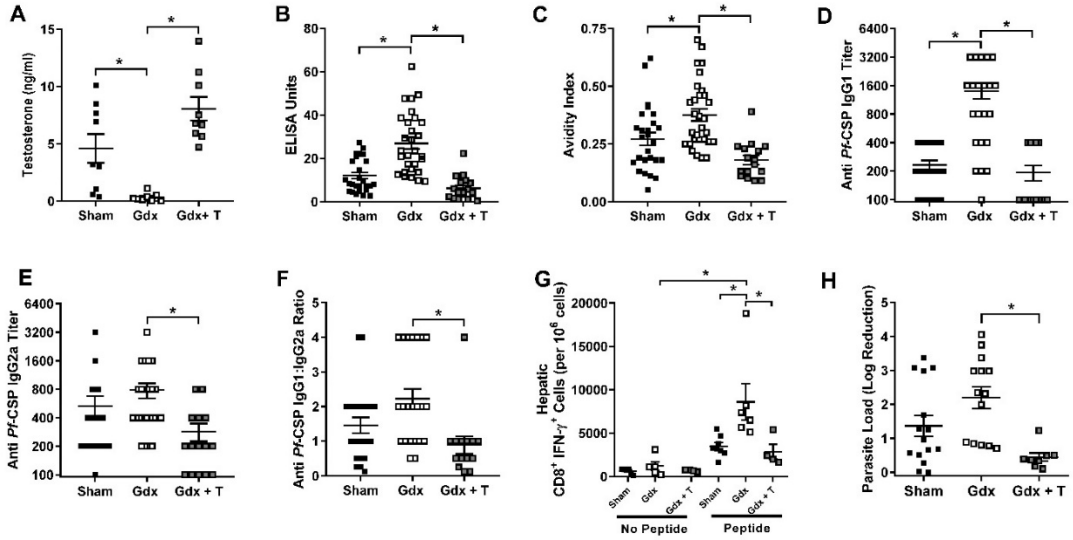
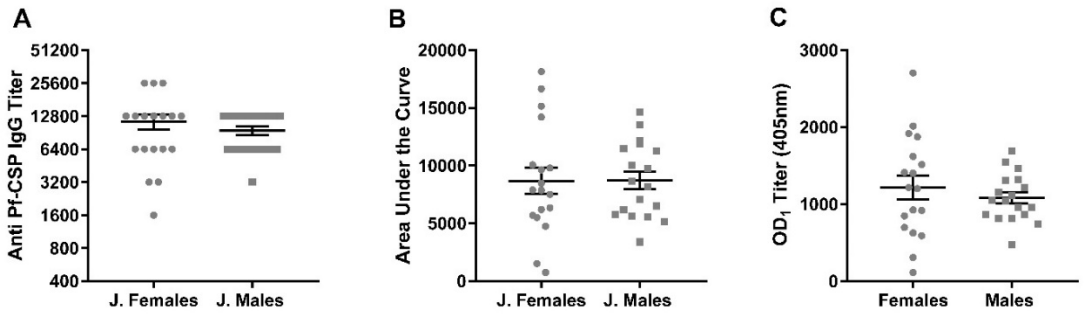

A
17β-Estradiol (pg/ml)
Sham
Ovx
B
ELISA Units
C
Avidity Index
D
Anti Pf-CSP IgG1 Titer
E
Anti Pf-CSP IgG2a Titer
F
Splenic CD8+ IFN-γ+ Cells (per 10^6 cells)
No Peptide
Peptide
G
Hepatic CD8+ IFN-γ+ Cells (per 10^6 cells)
H
Parasite Load (Log Reduction)

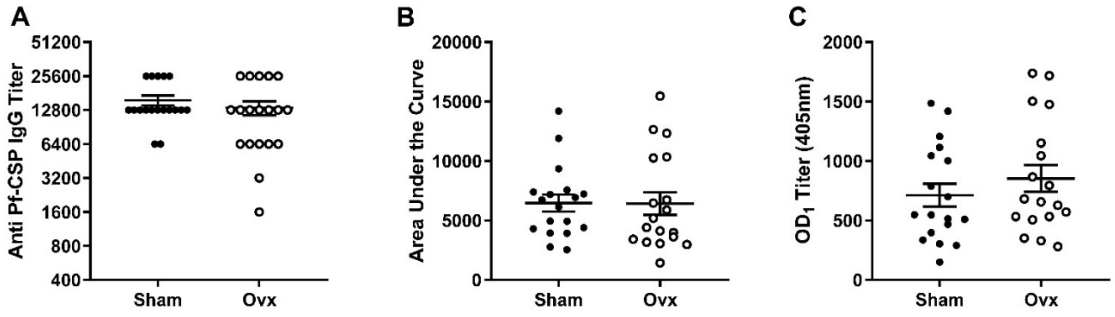
Figure 4.5


A
Testosterone (ng/ml)
Sham
Gdx
Gdx+ T
B
ELISA Units
C
Avidity Index
D
Anti Pf-CSP IgG1 Titer
E
Anti Pf-CSP IgG2a Titer
F
Anti Pf-CSP IgG1:IgG2a Ratio
G
Hepatic CD8+ IFN-γ+ Cells (per 10^6 cells)
No Peptide
Peptide
H
Parasite Load (Log Reduction)

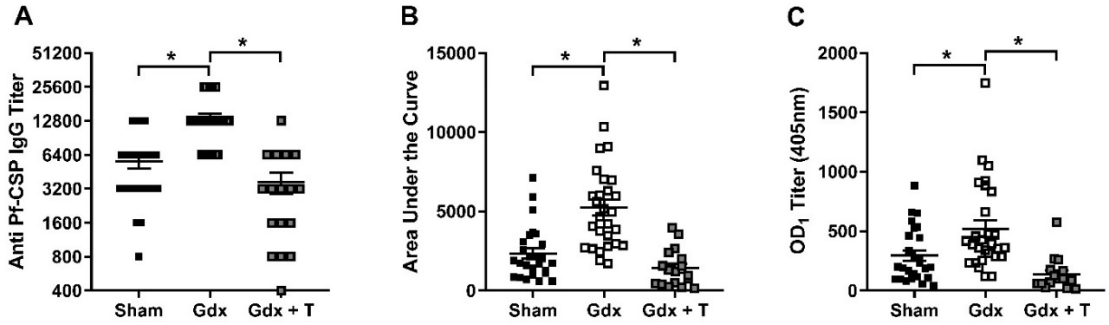
Supplemental Figure 4.1



Supplemental Figure 4.2



Supplemental Figure 4.3



Chapter 5

General Discussion

Landon G. vom Steeg

The research within this dissertation illustrates the need for a better understanding of how testosterone influences of the outcomes of infection and vaccination. Moreover, the findings of this dissertation demonstrate a novel role for testosterone in both reducing the severity of IAV infection males while inhibiting protective immunity to pre-erythrocytic stage malaria vaccination. These finding may have applications to other disease models and may provide novel insights leading to new infectious disease interventions and improvements in malaria vaccine design.

Testosterone confers protection against IAV mediated disease

Improperly regulated or persistent inflammatory responses can contribute to severe disease during IAV [267, 268, 274, 444]. In chapter 2, I made the unique observation that in hypogonadal males, whether age-related or surgically induced, testosterone treatment reduces the severity of IAV infection. Moreover, testosterone replacement in aged males reduced mortality following IAV infection. These effects were most pronounced during the recovery phase of infection (i.e., after virus had been cleared), and were associated with the delayed resolution of pulmonary inflammation, rather than changes in viral replication. These observations were expanded in chapter 3, where I showed that the delayed resolution of pulmonary inflammation and disease in testosterone depleted males was associated with the persistence of viral-specific $CD8^+$ T cells and the influx of eosinophils into lungs following control of viral replication. This is consistent with the literature which demonstrates an immunoregulatory role of testosterone [5, 23, 54-57]. As highlighted in the introductory chapter, the immune suppressive role of testosterone may be advantageous to overall male health as demonstrated the association of hypogonadism with increased all-cause mortality [35,

40-43]. The data contained within this dissertation suggest that testosterone replacement therapy may have additional benefits in the context of infectious disease.

Testosterone and not estrogen mediate sex differences following irradiated sporozoite vaccination

In the current study, depletion of female sex steroids did not alter either the adaptive immune response to vaccination or protection following parasite challenge. This is discordant with published literature showing both estradiol and progesterone to alter humoral immune responses and memory $CD8^+$ T cell formation [56, 166, 380, 431, 466-468]. Whether these differences in the effects of female sex hormone depletion represent differences in the context of vaccination versus infection, the route of inoculation, or differences in the pathways of immune activation warrant future study. Given that female mice were inherently more protected than males independent of female sex-hormone status, elucidation of the contributions other sex-specific factors (e.g., X-linked gene dosage) play in contributing to the more robust immune response in adult females relative to adult males warrants future study. As a notable example, several genes with immune function (e.g., CD40lg) identified as being associated with hepatic, but not splenic, memory $CD8^+$ T cell responses to irradiated sporozoite vaccination, are expressed on the X chromosome [472]. As adult females mounted greater hepatic, but not splenic CSP-specific $CD8^+$ T cell responses to irradiated sporozoite vaccination, whether the expression of these genes in females lymphocytes is greater due to escape from X chromosome inactivation should be considered [473].

In contrast with females, depletion of testosterone in males increased, whereas rescue of testosterone decreased, adaptive immune responses and protection in males following parasite challenge. Though the immunosuppressive properties of testosterone are

characterized, the impact of testosterone on vaccine responses has rarely been considered. Notably, in humans, elevated serum concentrations are associated with reduced humoral responses to the trivalent inactivated influenza vaccine [104]. Furthermore, as any malaria vaccination program will largely target pre-pubertal cohorts (i.e., infants and children), the suppressive effects of testosterone on pre-erythrocytic stage malaria vaccination may be largely inconsequential. Instead I propose, that understanding the mechanisms by which testosterone suppresses pre-erythrocytic stage vaccine efficacy will allow us to better inform malaria vaccine design. If we can identify the pathways that restrict vaccine efficacy, we can potentially exploit those pathways to enhance malaria vaccine efficacy. Moreover, future studies evaluating sex differences in RTS,S/ASO1 vaccine response and efficacy should be considered.

Testosterone replacement in aged males reduces mortality following IAV infection

In aged males, testosterone replacement reduced mortality and clinical severity, but had minimal effects on either morbidity or pulmonary inflammation. It is possible that increased survival in testosterone-treated aged males created a bias towards animals that were severely ill, but otherwise survived, masking differences between treatment groups during recovery from pulmonary inflammation at later time points during infection. Alternatively, other age-related physiologic changes may render the aged population more refractory to testosterone's protective effects. Consistent with this hypothesis, studies of androgen-dependent regulation of angiogenesis, found aging to be associated with reduced androgen responsiveness due to defective androgen receptor nuclear translocation in response to treatment [415]. Whether age-related defects in androgen signaling reduces the efficacy to testosterone treatment in our models warrants future consideration. Aging is also associated with profound dysregulation of the immune response to IAV, including delayed induction of both the innate and adaptive arms

of the immune system [474-477]. Given the effects of testosterone were most pronounced during the resolution phase of infection in young males, independent of changes in viral replication, it is possible that testosterone may not be able to resolve the issues of delayed immune induction in aged mice. Characterization of the effects of testosterone on the immune response to IAV in aged male will aid in elucidating the mechanisms by which testosterone reduces age-associated increases in mortality, but otherwise has minimal effects on morbidity and pulmonary inflammation.

Testosterone mitigates the accumulation of pulmonary eosinophils following IAV infection

Following control of IAV replication, the depletion of testosterone resulted in the accumulation of eosinophils in the lungs, which could not be explained by testosterone-associated changes in pulmonary concentrations of the type 2 cytokine (e.g., IL-5, IL-13, and eotaxin). Interestingly, previous studies in mice have also shown an accumulation of eosinophils in the lungs following control of IAV replication [445, 446], in the absence of detectable increases in IL-5 [446]. Although the precise role of eosinophils in the immune response to IAV are unclear, it has been suggested that that the accumulation of these cells represents the activation of type 2 tissue repair responses mediated by the production of the epidermal growth factor amphiregulin [446, 447]. Discordant with this hypothesis, our lab has previously reported, that though males are dependent on amphiregulin to repair damaged lung tissue faster than females, testosterone does not regulate the production of amphiregulin in response to IAV infection [302]. This would indicate that eosinophils may instead be contributing to immunopathology (e.g., eosinophilic pneumonia). Supporting this hypothesis, eosinophils have recently been shown to promote the proliferation and activation of $CD8^+$ T cells following IAV

infection in murine models of allergic asthma [217]. Furthermore, virus-specific $CD8^+$ T cells, which contribute to IAV-associated immunopathology [232, 277], also persisted in the lungs of testosterone-depleted mice in conjunction with eosinophilic infiltration.

Eosinophils are androgen responsive despite the absence of androgen receptor expression [24, 54, 83], with testosterone-mediated differences in eosinophilic airway responses instead being attributed to the actions Type II innate lymphoid cells (ILC2s) [88, 89]. Though not evaluated in this study, androgens have been shown to inhibit the maturation of ILC2s, while decreasing IL-5 production and eosinophilic responses in murine models of airway inflammation [88, 89]. Additional work to determine if testosterone is acting on ILC2 cells to inhibit eosinophilic following control influenza viral replication may have implications for other airway inflammatory diseases. Further, whether $CD8^+$ T cell cytotoxicity induced alarmin release is driving the eosinophilic response, as has recently been shown in a study of concanavalin A-induced hepatitis [478], should be considered.

Testosterone has also been shown to be protective against eosinophil associated inflammation in murine models of asthma and upper-airway inflammation [89, 438]. As mentioned above, testosterone reduces eosinophilic upper-airway inflammation by suppressing ILC2 numbers and their production of IL-5 and IL-13, by inducing intrinsic changes in these cells and through reductions in the expression of IL-33 and TSLP [89]. Additionally, testosterone suppresses house dust mite-induced eosinophilic inflammation and airway hyperresponsiveness through the suppression of IL-$17A^+$ Th17 cell numbers and their relative production of IL-23R mRNA [438]. *In vitro,* supraphysiological concentrations of testosterone (i.e., 50ng/ml but not 5ng/ml) reduce eosinophil viability and adhesion to human mucosal microvascular endothelial cells (HMMEC) through an unknown mechanism [87, 479]. Whether, testosterone ameliorates lower-airway eosinophil associated diseases (e.g., eosinophilic pneumonia and chronic

obstructive pulmonary disease), and non-airway eosinophilic associated diseases (e.g., atopic dermatitis, eosinophilic esophagitis, and eosinophilic colitis) has not been evaluated [480-482].

Testosterone does not alter the CD4$^+$ T cell response to IAV infection

Though the depletion of testosterone in male mice promoted the accelerated contraction of total CD4$^+$ T cell numbers, in response to testosterone, no differences in the numbers of Th1, Th2, or Th17 cells were observed as compared to placebo treated mice. In other inflammatory diseases, including experimental autoimmune encephalomyelitis, testosterone is associated with an expansion of Th2 and Th17 cell populations while suppressing Th1 activity [120, 122, 448]. Whether this represents differences between the strongly Th1-polarizing effects of IAV infection versus other inflammatory states should be considered. An alternate hypothesis is that single peptide stimulation of CD4$^+$ T cell in flow cytometry may not be adequate to capture the breadth of the protein-specific response of these individual cell types. The best way to further characterize polarization of CD4$^+$ T cells would be cytokine capture ELISpot analyses using virus-specific synthetic peptide pools as described by DiPiazza et al. [218, 483]. Additionally, regulatory T cells were increased at 21 days following IAV infection in testosterone-depleted males relative to testosterone replete male mice. This conflicts with testosterone treatment being demonstrated to promote the expansion in numbers and activation of regulatory T cells in murine models of inflammation [122-124]. This difference may be attributed to the greater pulmonary damage observed in testosterone depleted males, rather than an IAV specific response.

Testosterone promotes the contraction of CD8$^+$ T cells following control of IAV infection

The reduced severity of disease and pulmonary inflammation in testosterone-treated males following IAV infection was paralleled with the accelerated contraction of virus-specific CD8$^+$ T cells in the lungs, but not the spleen or mediastinal lymph nodes following control of viral replication. This observation is consistent with both the critical role IAV-specific CD8$^+$ T Cells play in controlling viral replication [231, 275, 276, 449], as well as their contribution to pulmonary immunopathology with prolonged or improperly regulated activation [232, 275, 277]. Both in humans and mice, testosterone alters the numbers, cytokine production, and proliferative potential of CD8$^+$ T cells [91, 113]. The significance of this tissue-specific effect is unknown, but whether these effects of testosterone on virus-specific CD8$^+$ T cells involve activation-induced cell death or inhibitory pathways warrants future study.

The dependence on androgen receptor signaling for accelerated contraction of virus-specific CD8$^+$ T cells and the expression of Ar in enriched splenic CD8$^+$ T cell populations suggested that testosterone might be acting directly on these CD8$^+$ T cell to mitigate IAV pathogenesis. Adoptive transfer studies were conducted and revealed that the presence of testosterone in the recipient mice was a better predictor of IAV outcome and contraction of virus-specific CD8$^+$ T cells than the presence of testosterone in the donor mice. These data suggest that testosterone is not acting directly on virus-specific CD8$^+$ T cells to induce intrinsic changes in these cells via androgen signaling. Instead, testosterone induces transient changes in these cells that are dependent on the presence of testosterone in the local environment in which they reside. Given the widespread expression of androgen receptors both in immune cells and epithelial cells in the lung [54, 451], testosterone may be acting indirectly on virus-specific CD8$^+$ T cells, through interactions with other cells to promote their contraction. If testosterone

in acting indirectly on $CD8^+$ T cells, then characterization of androgen receptor expression in the pulmonary environment during IAV infection may help identify potential interacting cell types. One such cell type may be eosinophils, which accumulated in the lungs of testosterone-depleted males in our model, and have recently been shown to promote the proliferation and activation of $CD8^+$ T cells following IAV infection in murine models of allergic asthma [217]. The use $CD8^+$ T cell lineage specific androgen receptor knock-out mice may be a better approach to further characterize whether testosterone is acting directly or indirectly to accelerate IAV-specific $CD8^+$ T cell contraction.

Testosterone exerts tissues-specific effects on antigen-specific $CD8^+$ T cells

In both Chapters 3 and 4, tissue-specific effects of testosterone on antigen specific $CD8^+$ T cell populations were observed, with testosterone promoting the contraction of virus-specific $CD8^+$ T cells in the lungs, but not in the in the spleen or mediastinal lymph nodes, following IAV infection, while testosterone reduced the numbers of CSP-specific $CD8^+$ T cells in the liver, but not spleen, following irradiated sporozoite vaccination. Though novel for $CD8^+$ T cells, these observations are consistent with previous reports of tissue-specific effects of testosterone on histamine production by mast cells in animal models, with testosterone depletion increasing histamine concentrations in the peritoneum, but not the lungs [484]. Moreover, testosterone and DHT have been demonstrated to have differential effects on vascular vasorelaxation based on localization within the host [485], suggesting that testosterone is able to exert regional effects within the same tissue type as well. Though the factors mediating these differences in androgenic effects are unclear, tissue-specific differences in testosterone concentration have been described in animal models [486, 487], with these differences at least in part being mediated by tissue-specific differences in the ability to uptake SHBG and albumin-bound

testosterone [488]. To date no murine studies have evaluated tissue-specific testosterone concentrations in the lungs or lymphatic system outside of the testes, and no studies have compared relative testosterone concentrations between the spleen and liver. Whether, the observed tissue specific effects of testosterone on $CD8^+$ T cells populations are mediated by local differences in testosterone concentration warrants future consideration. Furthermore, whether tissue specific interactions alter androgen receptor or androgen receptor-coregulator expression by $CD8^+$ T cells is also unknown but may provide an alternate mechanism explaining tissue specific effects of testosterone.

Though not explored in these studies, it is also possible that the observed tissue-specific effects of testosterone on $CD8^+$ T cell populations may be due to testosterone mediated changes in the expression of factors involved with the migration to, and persistence within, specific sites of infection and inflammation. For example, LFA-1/ICAM-1 interactions have been identified as critical to the retention of activated lymphocytes in the lungs [489, 490], while P-selectin glycoprotein ligand 1 (PSGL-1) is an adhesion molecular involved in the recruitment of $CD8^+$ T cells to the lungs in addition to playing a role in promoting T cells homeostasis [491-493]. Similarly, CCR5 and CXCR6 expression by T cells are required for the maintenance of memory liver antigen-specific T cells [494, 495], while ICAM-1 and VCAM-1 have been identified to play in role in the retention of T cells in the liver [496]. Though the effects of sex and sex hormones on T cell migration and adhesion molecule expression remains largely uncharacterized, observations that estrogen can inhibit PSGL-1 expression [497] and testosterone can modify TNFα mediated VCAM-1 expression by endothelial cells [498, 499], suggest that this may be an area warranting future study. Of specific interest would be sphingosine-1-phosphate and its receptors, which in addition to being involved in the trafficking of activated $CD8^+$ T cells to the liver in the context of

malaria [500], have recently been shown to be androgen responsive in other model systems [501, 502].

Testosterone and humoral immunity

The effects of testosterone on antibody responses to either infection or vaccination have rarely been considered, but where evaluated, testosterone has been reported to suppressive humoral immunity. Notably, in humans, neutralizing antibody production in response to influenza vaccination is inversely correlated with serum testosterone concentration [104], while testosterone treatment of human PBMCs reduces production of non-specific total IgG and IgM antibodies [92]. In contrast with these reports, in the influenza experiments detailed in Chapter 2, the manipulation of testosterone had no effect on either neutralizing or virus specific IgG titers following infection with the PR8 H1N1 influenza virus. As these findings were unexpected, these experiments were repeated using the ma2009 H1N1 influenza virus, and again both neutralizing and virus specific IgG titers were not altered with testosterone treatment. Whether these discordant findings reflect differences in androgen signaling between humans and mice, systemic immunization versus local pulmonary infection, or differences in the immunogen construct are unclear. However, in the experiments detailed in Chapter 4, the manipulation of testosterone had profound effects on anti-CSP specific total IgG and IgG1 titers following irradiated sporozoite vaccination. To date, no studies have evaluated the effects testosterone on memory B cell responses. Whether testosterone has differential effects on plasma B cells generated in response to acute primary infection versus the maintenance and recall of memory B cell responses requires future study.

Does testosterone act through dendritic or $CD4^+$ T cell populations to suppress irradiated sporozoite vaccine efficacy?

The suppression of both CSP-specific humoral and CSP-specific $CD8^+$ T cell responses by testosterone following irradiated sporozoite vaccination, suggest the possibility of a shared pathway in mediating these effects, with the actions of $CD4^+$ T cells and dendritic cells being two possible avenues. Antigen-specific $CD4^+$ T cells are important for the adequate priming and full development of $CD8^+$ T effector cell populations [503-506], in addition to being crucial for the survival and secondary expansion of memory $CD8^+$ T cell populations [504, 507-511]. $CD4^+$ T cell subsets also support the generation and maintenance of long lived plasma cells along with memory B cells [512-514], while cytokine production by $CD4^+$ T cells induces antibody class switching [515], and regulatory T cells have been shown to suppress productive interactions between Tfh and B cells during malarial blood stage infection [516]. Moreover, at least In rodent models, cytotoxic Th1 like $CD4^+$ T cells have been identified that are capable of directly controlling malaria liver stage infection through the removal of infection hepatocytes [514, 517, 518]. Similarly, antigen presenting dendritic cell subsets are also involved in the generation of both humoral and $CD8^+$ T cell responses to pre-erythrocytic malaria responses; often in conjunction or upstream of $CD4^+$ T cell responses. Notably, $CD8a^+$ and $CD11c^+$ dendritic cell populations contribute to $CD8^+$ T cell priming and activation [500, 511, 519, 520], while dendritic cells contribute to antibody mediated protection through the direct presentation of antigen to B cells in addition to the induction of the above mentioned $CD4^+$ T cell help [521, 522]. Any potential role for testosterone in altering dendritic or $CD4^+$ T cell responses to irradiated sporozoite vaccination were not evaluated in this dissertation. However, despite testosterone not acting through either population in the context of IAV infection, data from other disease model systems, as summarized in chapter 1, strongly suggest that testosterone can alter both

the proinflammatory and immunosuppressive responses of these two relatively diverse cell types. Furthermore, despite the potential implications on antibody mediated protection, to date no study has yet directly evaluated the effects of testosterone on Tfh cell populations. Whether testosterone is altering Tfh cell responses to irradiated sporozoite vaccination should be considered.

Testosterone alters tolerance, but not resistance to IAV infection

In response to microbial challenge, hosts can employ one of two distinct evolutionary strategies to ensure survival: tolerance and resistance [523-525]. Resistance to infection is traditionally defined as the ability of the host to kill or expel a pathogen or otherwise inhibit its reproductive success [523-525]. This strategy is dependent on the robustness of both the innate and adaptive immune response to the pathogen [526]. Because of the immune system's efforts to eliminate the pathogen, substantial host tissue damage or loss of function can consequently occur [527-529]. Disease tolerance, however, reduces the fitness costs of infection independent of changes of pathogen survival or burden [526, 527]. Through tolerogenic responses, the consequences of either pathogen or immune-mediated damage can be ameliorated, subsequently maintaining host health [526-529]. Moreover, these strategies are not mutually exclusive, with many of the same immune system components contributing to both host resistance and tolerance [524, 525, 530]. For example, Th2-associated alternatively activated macrophages are involved in helminthic parasite killing, in addition to the repair of helminthic parasite-induced tissue damage [531].

In the context of IAV infection, testosterone did not alter resistance to infection as both testosterone-depleted and testosterone-replete males maintained similar viral replication kinetics. Testosterone instead improved tolerance of IAV infection (i.e., alleviated body mass

loss and hypothermia) through reductions in the numbers of pulmonary eosinophils and virus-specific $CD8^+$ T cells following control of viral replication. Thus, testosterone accelerated the resolution of pulmonary inflammation and reduced immunopathology in young males in the absence of immune alteration that interfered with control of viral replication. When advanced age was added as confounder, though aged males were slower to clear virus, no changes in viral replication were observed with testosterone treatment. However, in contrast with young males, testosterone failed to reduce body mass decline or hypothermia in aged males, while significant improvements in both survival and clinical severity were still observed. The data from these studies demonstrate that the protective effects of testosterone on IAV infection are mediated by improvements in host tolerance and resilience (i.e., the ability to recover from disease [532]), rather than through alterations to resistance type responses.

The broader evolutionary impacts of testosterone on the immune response

Observations that testosterone both regulates male secondary sexual trait development in addition to suppressing the immune response has led to the development of the immunocompetence handicap hypothesis (ICHH) [533]. This hypothesis proposes that since testosterone imposes an immunological fitness cost, only high fitness or resistant (i.e., able to remain pathogen free) males can maintain energetically costly testosterone mediated secondary sexual traits in the presence of strong immunological pressure [533-535]. Moreover, when intra-host resources are limited, lower fitness males will be unable to afford the energetic costs of maintaining immunocompetence simultaneously with those of testosterone mediated sexually selective traits. In support of this hypothesis, several studies using vertebrate animal models have demonstrated a correlation between reduced parasite load, or the ability to cope with high parasite loads, and the expression of testosterone mediated secondary sexual traits [536-538].

Similarly, the data presented in this dissertation and by others, abundantly demonstrate that testosterone can suppress the immune response to both vaccination and infection [5, 23, 104, 106, 121]. The individual consequences of this testosterone-mediated immune suppression are likely to depend on the nature and severity of the immunological challenge and the magnitude of sexual selection.

The ICC hypothesis also proposes that the interactions between testosterone and the immune system are dynamic and bidirectional, with the temporary suppression of testosterone being an mechanism to overcome infection in the presence of sexual selection [533, 539]. Specifically under the pressure of substantial immunological challenge, the fitness costs imposed by the infection will overwhelm the fitness benefits of maintaining secondary sexual characteristics, resulting in a decline in circulating testosterone concentrations [533, 539]. In agreement with this hypothesis, In chapter 2, I demonstrate that IAV infection induces a transient decline in testosterone concentration in young male mice corresponding with the onset of disease, while no decline in the relatively lower testosterone concentrations were observed in aged male mice approaching reproductive senescence [432, 540]. These findings are consistent with the observations of others demonstrating that immune activation, irrespective of the source, can suppress endogenous testosterone production [539, 541-544].

However, variation in the vertebrate immunological response to testosterone suggest that the evolutionary consequences of testosterone-immune interactions may be more complex. In contrast with the findings of Furman *et. al*., a recent report in young, affluent, and healthy males [5, 23, 106, 121], found no evidence in support of the assumptions of the ICC hypothesis following inactivated influenza vaccination, with free testosterone and DHT instead being positively correlated with the magnitude of antigen-specific IgG antibody responses at 4 weeks post vaccination. Similarly, in avian populations, experimental treatment of captive bird

populations with testosterone has been observed to suppress antibody responses to immunization with sheep red blood cells (SRBC), while in free-living males, testosterone was positively correlated with the robustness of the antibody response to SRBC immunization [536]. These observations along with others, have led to the proposal of a multitude of potential confounders and environmental interactions, including the nature of the immunological challenge, differences in vertebrate system biology, immune parameters measured, the condition of the host (e.g., age, reproductive status, presence of comorbidities), interactions with glucocorticoid stress hormones, and the potential conversion of testosterone to estrogen [534, 545-549]. Furthermore, it is likely that the expression of testosterone doesn't incur a fitness cost under conditions of high resource availability and low competitive stress, where adequate intra-host resources allow for robust expression of simultaneous immunocompetence and secondary sexual characteristics. Whether the potential metabolic savings of testosterone mediated immune suppression would outweigh the metabolic costs of any resulting infection has also been challenged [550].

An alternate hypothesis is that rather than being broadly immunosuppressive, as assumed by the ICH hypothesis, testosterone instead acts to modify and/or redistribute components and responses of the immune system [550]. With this idea, testosterone selectively modulates individual components of the immune system, in response to host status and environmental stimuli. Furthermore, as with the ICH hypothesis, these interactions would be transient and bidirectional to maximize reproductive success. Consistent with this hypothesis, testosterone has been observed to enhance neutrophil and mast cells numbers and responses [551, 552], while promoting platelet aggregation [553-555]. It is worth noting, that for many species, testosterone promotes aggressive interactions and the potential for wounding, while simultaneously promoting immune system components (e.g., mast cells, neutrophils, and

platelet aggregation) involved in early responses to wounding [556-561]. In contrast, testosterone generally, but not always, suppresses B cell, Th2, and Th17 associated immunity, while reducing the NK cell activity and the production of proinflammatory mediators by macrophages [90, 561-564]. If testosterone has evolved a role of limiting the energetic cost of the immune system in favor of reproductive success, then the ability to selectively alter the immune system in response to specific stimuli would reduce the fitness costs associated with secondary sexual traits. In accordance with this idea, I did not observe testosterone to broadly suppress the immune response to IAV infection. Instead testosterone suppressed eosinophilic and virus-specific $CD8^+$ T cell responses only following control of viral replication, while neutrophil numbers were transiently increased, and other immune parameters were not altered. Similarly, though testosterone suppressed both antibody and CSP-specific $CD8^+$ T cell responses following irradiated sporozoite vaccination, it is possible that in the absence of previous exposure, the immunological challenge of an essentially killed vaccine, was insufficient to warrant energetic resource reallocation. Whether intra-cage social hierarchy continues to modify testosterone associated responses could provide additional insight into the relationships between testosterone and the evolution of sexual selection

The impact of testosterone on infectious disease

The relationships between testosterone and the outcomes of infectious disease appear to be complex. While in Chapter 2 and Chapter 3, I show testosterone-induced changes in immune function to be protective against severe disease following IAV infection, in other animal models of infection, testosterone has been shown to worsen disease outcomes. Notably in rodent models of amoebic infection [114, 143, 154], *Babesia* infection [155], *Leishmania* infection [157, 158], mycobacterium infection [160, 161], and with malaria [381, 385-389],

testosterone has been shown to increase infection severity in association with increased pathogen load. I hypothesize that when disease following infection is due the ability to control the pathogen, testosterone will likely be detrimental. Conversely, in infections where disease is largely attributable to the immune response to infection, as with sub-lethal IAV infections [54, 267], the immunosuppressive effects of testosterone will likely be protective. To date, few clinical studies have evaluated the impact of testosterone on infections; including infection with IAV. Whether testosterone in males increases the risk of secondary infection in males, warrants future study.

Conclusion

The overall impact of this dissertation resides in its novelty. Despite the increasing numbers of hypogonadal men, and the widespread use of testosterone replacement therapy, very little work has evaluated the effects of testosterone on the outcomes of infection. No studies to date have considered the effects of testosterone on the efficacy of pre-erythrocytic stage malaria vaccination. With this research, I hope to raise awareness of the potential impacts of testosterone on the outcomes of infection and vaccination. Although the exact mechanisms by which testosterone mediates these effects deserve to be further explored, understanding the mechanisms by which testosterone suppresses vaccine efficacy may provide insight leading to improvements in malaria vaccine design. Furthermore, these findings suggest that testosterone may have therapeutic potential in treatment of infectious disease.

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Curriculum Vitae

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Education

Johns Hopkins Bloomberg School of Public Health - Baltimore, MD **2013 – Present**
Ph.D. Candidate in Molecular Microbiology and Immunology
Laboratory of Dr. Sabra Klein

Tulane University School of Public Health and Trop. Medicine - NOLA, LA **2010 – 2012**
M.S.P.H. in Tropical Medicine

Portland State University - Portland, OR **2004 – 2006**
B.S. in Biology *(cum laude)*

Central Oregon Community College - Bend, OR **2000 – 2003**
A.S. in Exercise Science

Experience

Graduate Research Assistant **2013 – Present**
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Advisor: Sabra Klein, Ph.D.

- Performing research on the effects of the sex hormone testosterone on influenza pathogenesis as well as sex differences in the immune responses to malaria vaccination.
- Gained expertise in standard endocrinology, immunology, and virology methods, including survival surgery, flow cytometry, cell culture, and in vivo cell manipulation.

Medical Research Technician **2012 – 2013**
Tulane University School of Medicine, New Orleans, LA
Supervisor: John Schieffelin, M.D.

- Conducted research evaluating the epidemiology of viral infections in Sierra Leone, and the isolation and characterization of flavivirus specific monoclonal antibodies.
- Became proficient with cell and virus culture methods, including use of reporter viruses and ELISAs.

MSPH Practicum **2011**
U.S. Naval Medical Research Unit – 6, Tumbes, Peru
Supervisor: Daniel Bausch, M.D.

- Assisted with epidemiologic field studies concerning influenza virus transmission, vector-borne disease control, and the etiology of undifferentiated febrile illnesses.
- Gained exposure to field survey methods as well as diagnostic sample collection and processing.

Graduate Research Assistant **2010 - 2012**
Tulane University School of Public Health and Tropical Medicine, New Orleans, LA
Supervisor: Daniel Bausch, M.D.

- Assisted with manuscript preparation and data management.
- Became proficient with the use of Endnote and the establishment and maintenance of long-term research data bases.

Laboratory Assistant **2009 - 2010**
Colorado State University College of Veterinary Medicine and Biomedical Sciences
Supervisor: William Black, Ph.D.

- Assisted senior staff with research concerning molecular markers of insecticide resistance in the viral vector *Aedes aegypti*.
- Gained proficiency with the use of PCR and DNA sequencing.

Lead Forestry Technician – Wildland Fire Module Lead **2009**
Rocky Mountain National Park, Estes Park, CO
Supervisor: Matt Dutton

- Provided supervision and leadership during all fire management operations for the 12-member Rocky Mountain National Park Fire Module.
- Expanded my knowledge of field methods used in the study of fire ecology and fire effects. Gained proficiency with the use of GIS based mapping methods and the use of BEHAVE to model fuel types and perform predictive fire behavior analyses.

Lead Forestry Technician – Assistant Fire Engine Captain **2008**
Rocky Mountain National Park, Estes Park, CO
Supervisor: Matt Dutton

- Provided supervision and leadership during all fire management operations for the park's 3-member wildland fire engine crew. Served as the Acting Fire Station Captain for the duration of the fire season.
- Gained further experience with leadership during critical incidents and the mentoring of junior fire staff.

Teaching and Mentoring Experience

Teaching Assistant: Evolution of Infectious Diseases, 2016, 2017, and 2018
Public Health Perspectives on Research, 2017
Tropical Virology, 2012

Academic Mentoring: Daniel Smith, PhD Rotation Student, 2018
Megan Wood, PhD Rotation Student 2018
Kitty Guo, WISE High School Student Intern, 2017 - 2018
Kimberly Rousseau, PhD Rotation Student, 2017
Eric Sasse, Undergraduate Research Assistant, 2016 - 2017
Jamiaha Thomas, High School Summer Intern, 2015 - 2017
Kyla Britson, PhD Rotation Student, 2015
Meghan Vermillion, PhD Rotation Student, 2014
Ornob Alam, ScM Rotation Student, 2014

Honors and Awards

Jane Welsh Russell Fellowship in Molecular Microbiology & Immunology, JHBSPH	2018
American Society for Virology Travel Grant	2017
The Katharine E. Welsh Fellowship in Immunology & Infectious Disease, JHBSPH	2017
CEIRS Intramural Training Fellowship	2017
Carlton & Estelle Herman Award in Parasitology, Vector Bio, & Animal-Borne Dis., JHBSPH	2014
Dean's Scholarship, Tulane University School of Public Health & Tropical Medicine	2010
STAR Award, Rocky Mountain National Park Fire Management	2009
STAR Award, Rocky Mountain National Park Fire Management	2008
Lassen Volcanic National Park Superior Performance Award	2007
NPS Unit Citation for Exceptional Performance During the 2005 Ward Shooting	2006

Professional Service

Ad Hoc Reviewer

Nature Communications, 2018
PloS Pathogens, 2017
Journal of Virology, 2017
The Journal of Infectious Diseases, 2016
The Journal of Immunology, 2016
PLoS One, 2015
Human Genetics, 2015

Research Interests

Evolution of Infectious Diseases, Host-Pathogen Dynamics, Infectious Disease Pathogenesis, Disease Ecology, Zoonotic Diseases, Emerging Infectious Diseases, Vector-Borne Diseases.

Publications

vom Steeg LG, Flores-Garcia Y, Zavala F, Klein SL. Irradiated sporozoite vaccination induces sex-specific immune responses and protection against malaria in mice. *Submitted to Vaccine.*

vom Steeg LG, and Klein SL. Sex and sex steroids impact influenza pathogenesis across the life course. *Seminars in immunopathology*. 2019 Mar;41(2):189-194. doi: 10.1007/s00281-018-0718-5

Vermillion MS, Ursin RL, Kuok DIT, **vom Steeg LG**, Wohlgemuth N, Hall, OJ, Fink AL, Sasse E, Nelson A, Ndeh R, McGrath-Morrow S, Mitzner W, Chan MCW, Pekosz A, Klein SL. Production of amphiregulin and recovery from influenza is greater in males than females. *Biology of sex differences*. 2018 Jul 17;9(1):24. doi: 10.1186/s13293-018-0184-8

Vermillion MS, Nelson A, **vom Steeg LG**, Loube J, Mitzner W, Klein SL. Pregnancy preserves pulmonary function following influenza virus infection in C57BL/6 mice. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2018. doi: 10.1152/ajplung.00066.2018.

- American Physiological Society Select Article

vom Steeg LG, and Klein SL. Sex Steroids mediate bidirectional interactions between hosts and microbes. *Hormones and Behavior.* 2017. Feb;88:45-51. doi: 10.1016/j.yhbeh.2016.10.016

Potluri T, Engle K, Fink A, **vom Steeg LG**, and Klein SL. Sex reporting in preclinical microbiology and immunological research. Invited commentary for *mBio*. 2017 Nov 14;8(6). doi: 10.1128/mBio.01868-17

vom Steeg LG, and Klein SL. SeXX matters in infectious disease pathogenesis. *PLoS Pathogens*. 2016. 12(2):e1004374. doi: 10.1371/journal.ppat.1005374

- 2016 PLoS Pathogens top 50 most downloaded article

vom Steeg LG, Vermillion MS, Hall OJ, Alam O, McFarland R, Chen H, Zirkin B, Klein SL. Age and testosterone mediate influenza pathogenesis in male mice. *American Journal of Physiology Lung Cellular and Molecular Physiology*. 2016. Dec 1;311(6):L1234-L1244. doi: 10.1152 /ajplung.00352.201

Presentations

Oral Presentations

vom Steeg LG, Wood MK, Zirkin B, Klein SL. 2018. Testosterone protects against severe influenza by contracting CD8+ T cell responses after viral clearance. Oral presentation at the 37th annual meeting of the American Society for Virology, College Park, Maryland.

vom Steeg LG, Klein SL. 2017. Advanced age and low testosterone delay the resolution of CD8+ T cell responses following influenza virus infection in male mice. Oral presentation at the 36th annual meeting of the American Society for Virology, Madison, Wisconsin.

vom Steeg LG, Vermillion MS, Hall OJ, Klein SL. 2016. Age and testosterone shift virus-specific CD8+ T cell and regulatory T cell responses during influenza virus infection in male mice. Selected oral presentation at the 36th annual meeting of the American Society for Reproductive Immunology, Baltimore, Maryland.

vom Steeg LG, Vermillion MS, Hall OJ, Alam O, McFarland R, Chen H, Zirkin B, Klein SL. 2016. Age and testosterone affect influenza pathogenesis in male mice. Oral presentation at the 35th annual meeting of the American Society for Virology, Blacksburg, Virginia.

Poster Presentations

vom Steeg LG, Klein SL. 2017. Testosterone protects against severe influenza by promoting the contraction of CD8+ T cell responses following viral clearance. Poster presented at 11th Annual CEIRS Network Meeting, New York City, New York.

- Awarded best poster

vom Steeg LG, Flores-Garcia Y, Zavala, FP, Klein SL. 2017. Sex Predicts the efficacy of immunization with irradiated plasmodium sporozoites in adult mice. Poster Presented at the 66th annual meeting of the American Society of Tropical Medicine & Hygiene, Baltimore, Maryland.

vom Steeg LG, Klein SL. 2017. Advanced age and low testosterone delay the resolution of CD8+ T cell responses following influenza virus infection in male mice. Poster presented at the 2017 NIA//NIAID Aging and Immunity Symposium, Rockville, Maryland.

vom Steeg LG, Flores-Garcia Y, Rousseau KE, Zavala, FP, Klein SL. 2017. Sex Predicts the efficacy of immunization with irradiated plasmodium sporozoites. Poster Presented at the 2017 Malaria Vaccine Symposium, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland.

vom Steeg LG, Flores-Garcia Y, Rousseau KE, Zavala, FP, Klein SL. 2017. Sex Predicts the efficacy of immunization with irradiated plasmodium sporozoites. Poster presented at Vaccine Day, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland.

vom Steeg LG, Hall, OJ, Alam O, Vermillion M, McFarland R, Zirkin B, Klein SL. 2015. Testosterone regulates the outcome of influenza by altering the immune responses in male mice. Poster presented at the 34th annual meeting of the American Society for Virology, London, Ontario, Canada.

Alam O, **vom Steeg LG**, Hall OJ, Klein SL. 2015. Age-related reductions in testosterone and the impact on influenza A virus infection in male mice. Poster presented at the 8th annual Research on Aging Showcase, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland. *1st place in student poster category*.

Quandelacy TM, Johnson J, Kessler M, **vom Steeg LG**, Craig I, et al. 2015. Comparative Analysis of Seasonality of Childhood Infections in Seven United States Cities in the Pre-Vaccination era. 143rd APHA Annual Meeting and Exposition, Chicago, IL, USA.